Organophosphate Compound Degradation Using Enzyme Immobilized Carriers

M.Sc. Thesis

By

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DEPARTMENT OF BIOSCIENCES AND BIOMEDICAL ENGINEERING

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A THESIS

Submitted in partial fulfillment of the requirements for the award of the degree
of
Master of Science

by

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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **ORGANOPHOSPHATE COMPOUND DEGRADATION USING ENZYME IMMOBILIZED CARRIERS** in the partial fulfillment of the requirements for the award of the degree of **MASTER OF SCIENCE** and submitted in the **DEPARTMENT OF BIOSCIENCES AND BIOMEDICAL ENGINEERING, Indian Institute of Technology Indore**, is an authentic record of my own work carried out during the time period from July 2024 to May 2025 under the supervision of Dr. Abhijeet Joshi, Associate Professor, BSBE, IIT Indore.

The matter presented in this thesis has not been submitted by me for the award of any other degree of this or any other institute.

Yashi Singh 19/5/25

Signature of the student with date
(Yashi Singh)

This is to certify that the above statement made by the candidate is correct to the best of my/our knowledge.

19/5/25

Signature of the Supervisor of M.Sc. thesis

(Dr. Abhijeet Joshi)

Yashi Singh has successfully given her M.Sc. Oral Examination held on **06.06.2025**

19/5/25

Signature of Supervisor of MSc thesis
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Convener, DPGC
Date: 19/05/2025

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Yashi Singh

Dedicated to my Parents

Mr. Rajeev Kumar,

Mrs. Babita Rani

and my brother

Yash

Abstract

Pesticides are widely used in agriculture to protect crops from pests and increase yields. However, their use has been associated with adverse environmental and health effects due to organophosphate (OP) pesticides. OP poisoning has been linked to various symptoms, including neurological disorders and death. To address these issues, there is a growing interest in developing methods for detecting pesticides and the remediation of contaminated soils and water. The present study investigates the enzymatic degradation of various OP Pesticides. Investigating the use of immobilized enzymes in bioremediation processes for the degradation of OP pesticides and the real-life application of enzymes degrading OP-based Pesticides.

The research involved discovering a new substrate being degraded by OP hydrolyzing enzymes. The enzyme was also immobilized onto Sodium Alginate Nanoparticles, Gelatin Nanofibre and Cloth. These immobilized enzymes were then tested for their ability to degrade OP pesticides. The results showed that the immobilized enzymes effectively degraded OP pesticides. The efficiency of the immobilized enzymes was found to be dependent on several factors, including the type of enzyme, the immobilization method, and the characteristics of the contaminated environment. The research also evaluated the stability and reusability of the immobilized enzymes and found that they retainedtheir activity after several cycles of use.

Overall, the findings of this study suggest that immobilized enzymes can be a promising tool for the bioremediation of OP pesticide-contaminated environments offering several advantages, including increased stability and reusability, and can provide an environmentally friendly and cost-effective approach for removing OP pesticides. The research also highlights the need to optimize further and develop immobilized enzyme-based bioremediation strategies to clean contaminated environments effectively, beneficial for farmers.

LIST OF PUBLICATIONS

From Thesis

Biomimetic Approach of using Gelatin Nanofibre in Organophosphate Compound Degradation (Manuscript under preparation)

Apart from the Thesis

Role of Cancer Cell-Derived Exosomal Glycoproteins in Macrophage Phenotypic Change. (accepted in **Molecular Biology Reports**)

The Effect of Cancer Cell-Derived Exosomal Proteins on macrophage Polarisation. (published in **Experimental Cell Research 2024**)

Recent Developments in CRISPR-Cas Systems for Human Protozoan Diseases (published in **Progress in Molecular Biology and Translational Science**)

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ABBREVIATIONS

AChE	Acetylcholine Esterase
OPAA-FL	Organophosphorus Acid Anhydrases
OPH	Organophosphorus Hydrolase
OPs	Organophosphorus Compounds
PTE	Phosphotriesterase
FITC	Fluorescein Isothiocyanate
BSA	Bovine Serum Albumin
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
DMEM	Dulbecco's Modified Eagle Medium
PPM	Parts Per Million
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
LOD	Limit of Detection
PBS	Phosphate buffered saline
Ni NTA	Nickel Nitriloacetic Acid
Nus-A	N-Utilization Substance Protein A
OC	Organochlorine
DMSO	Dimethyl Sulfoxide

Chapter 1

Introduction

1.1. Pesticides: A Universal Risk

Pest infections are the major challenges in agriculture, causing significant crop losses globally. Approximately 40% of crop production is lost annually due to pests. To diminish these losses, pesticides are extensively used in agriculture. These chemicals help control weeds, insects, and diseases, thereby ensuring food security and enhancing crop yields. However, the widespread use of pesticides has raised concerns about human exposure (Oerke, 2006). Pesticides are substances used to prevent, destroy, or control pests. They include a wide range of chemicals, each designed to target specific pests. The primary goal of using pesticides is to protect crops from pests and diseases, which in turn helps in increasing agricultural productivity. However, the extensive use of pesticides has led to environmental contamination and potential health risks for humans and animals (Pimentel, 2005). Pesticides exposure to humans can occur through various means, including ingestion of contaminated food and water, inhalation of pesticide sprays, and dermal contact. Chronic exposure to pesticides has been linked to various health issues, including respiratory problems, skin conditions, and even cancer. Therefore, understanding and mitigating pesticide exposure is crucial for public health (Alavanja *et al*., 2004).

Pesticides are classified into several categories based on their chemical structure and mode of action. The four main classes include Organochlorines, Triazines, Carbamates and Organophosphates. Organochlorines are chlorinated hydrocarbons used prominently in the past. They are persistent in the environment and can bioaccumulate in the food chain, leading to long-term ecological and health effects.

Triazines are herbicides used to control broadleaf weeds and grasses. They are less persistent than organochlorines but can still contaminate water sources. Carbamates are pesticides that inhibit acetylcholinesterase, an enzyme essential for nerve function. They are used to control insects and nematodes.

Organophosphates are widely used insecticides that also inhibit acetylcholinesterase. They are known for their acute toxicity and potential to cause neurotoxic effects (George *et al*., 2004). Organophosphates (OPs), once known by terms like organic phosphates, phosphorus insecticides, and nerve gas relatives, are derived from phosphorus acids and are among the most toxic pesticides for vertebrates. Their chemical structure closely resembles that of nerve agents, leading to similar modes of action. The insecticidal properties of OPs were first recognized in Germany during World War II, while researching highly toxic nerve agents such as sarin, soman, and tabun. This research began as an effort to find alternatives to nicotine, which was widely used as an insecticide but was scarce in Germany at the time.

Two main characteristics define organophosphates. First, they tend to be more toxic to vertebrates compared to other insecticide classes. Second, they are generally unstable or nonpersistent. This instability made them attractive as alternatives to the more persistent organochlorines in agricultural applications. In response to concerns about their toxicity, the Environmental Protection Agency (EPA) initiated a comprehensive review of OPs under the Food Quality Protection Act (1996), starting in the late 1990s. This led to the voluntary cancellation of many OP products and restrictions on others (Adeyinka *et al*., 2024). Organophosphates (OPs) work by blocking a key

enzyme in the nervous system called cholinesterase (ChE). The binding of OP compounds to acetylcholinesterase (AChE) primarily involves the amino acids such as serine, histidine, and glutamate which form catalytic triad at the enzyme's active site as shown in **Fig. 1.1.** These amino acids are crucial for the enzyme's activity and is targeted by OP compounds upon phosphorylation, leading to inhibition of AChE (Alexandre *et al*., 2021) which leads to a buildup of acetylcholine (ACh) at the junctions between nerve cells and between nerves and muscles. As a result, this causes rapid muscle twitching and ultimately leads to paralysis.

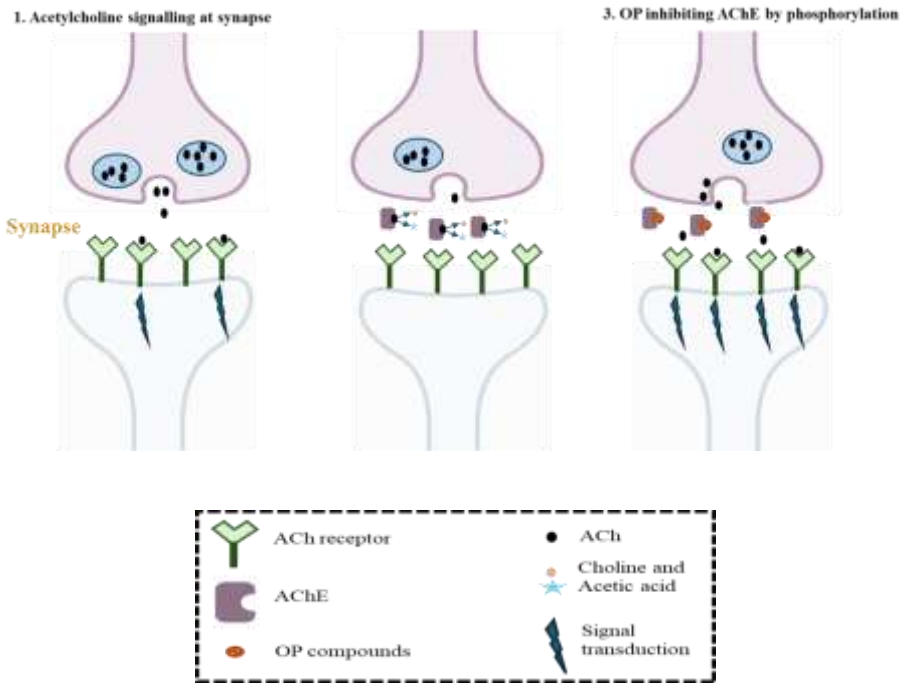

Fig. 1.1. Mechanism of action of OP compounds on inhibition of Acetylcholine esterase enzyme. (Biorender)

1.2. Classification of OP compounds

Organophosphates (OPs) are esters of phosphorus that have different combinations of oxygen, carbon, sulfur, and nitrogen. This leads to six distinct subclasses: phosphates, phosphonates, phosphorothioates, phosphorodithioates, phosphorothiolates, and phosphoramidates. Generally, OPs can be categorized into three main groups: aliphatic, phenyl, and heterocyclic derivatives (Adeyinka *et al*., 2024):

1.1.1. Aliphatic Organophosphates:

Aliphatic OPs have a structure resembling carbon chain. The first OP used in agriculture was tetraethyl pyrophosphate (TEPP), introduced in 1946, which falls into this category. Other notable examples include malathion as shown in **Fig. 1.2.**, trichlorfon (Dylox®), etc.

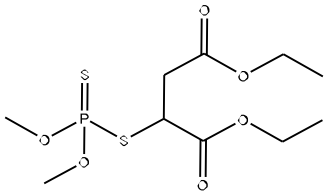

Fig. 1.2. Structure of malathion

1.1.2. Phenyl Derivatives: Phenyl organophosphates (OPs) feature a phenyl ring, where one hydrogen atom is replaced by a phosphorus moiety, while other hydrogens may be substituted with chlorine, nitro, methyl, cyanide, or sulfur groups. Generally, phenyl OPs are more stable than their aliphatic counterparts, resulting in longer- lasting residues. The first phenyl OP used in agriculture was parathion (ethyl parathion) as shown in **Fig. 1.3.**, introduced in 1947. Other examples include methyl parathion, profenofos (Curacron®), sulprofos

(Bolstar®), isofenphos (Oftanol®, Pryfon®).

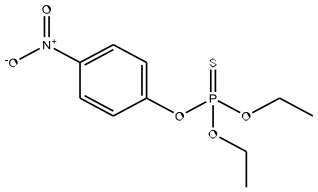

Fig. 1.3. Structure of ethyl parathion

1.1.3. Heterocyclic Derivatives: Heterocyclic organophosphates (OPs) are characterized by ring structures made up of different types of atoms, such as oxygen, nitrogen, or sulfur. The first OP in this category was diazinon, introduced in 1952. Other notable examples include azinphos-methyl, chlorpyrifos as shown in **Fig. 1.4.** (methidathion (Supracide®), phosmet (Imidan®) (George *et al*., 2004).

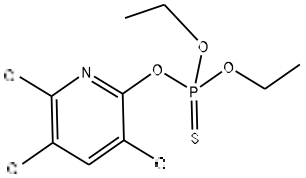

Fig. 1.4.: Structure of chlorpyrifos

OP compounds are also commonly known as "phosphotriesters" because of the presence of three phosphodiester linkages. Structurally OP compounds are composed of a phosphorous atom which either form double bond with oxygen or sulphur. R stands for different side chains where R^1 and R^2 groups could be aryl or alkyl groups, halogens, esters or amides. X is the leaving group (**Fig. 1.5.)** which is susceptible towards hydrolysis, replaced when OPs phosphorylate acetylcholinesterase (AChE), targeting the serine in the enzyme's active site (Jaiswal *et al.*, 2024).

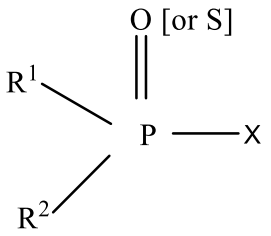

Fig. 1.5. General structure of OP compounds

Degradation of organophosphate compounds is essential for reducing their harmful effects. Conventional methods involve the use of bacteria or enzymes to break down these compounds. Bacteria such as *Pseudomonas* and *Flavobacterium* have been shown to degrade organophosphates effectively. Enzymes like organophosphorus hydrolase (OPH) and phosphotriesterase (PTE) are also used to catalyze the hydrolysis of organophosphates, leaving them non-toxic (Singh *et al*., 2006)(Mulbry & Karns, 1989)(Qin *et al*., 2024). Advances in genetic engineering have further enhanced the degradation of organophosphates. By modifying microbes to overexpress specific enzymes, there is improvement in the efficiency of organophosphate degradation. For example, genetically engineered *Escherichia coli* strains that overproduce OPH have shown increased degradation rates of organophosphate compounds (Ali *et al*., 2012; Priyanka *et al*., 2023).

This research aims to address challenges caused by Organophosphate Compounds by exploring innovative method for degrading and detoxifying OP compounds. The objective is to make Gelatin Nanofibres cross-linked with enzyme degrading OP compound into less toxic constituents. Gelatin nanofibers are made from biocompatible polymer, gelatin, a protein derived from collagen, a natural polymer found in animal tissues and is known for its biocompatibility and biodegradability. Gelatin nanofibers (GNFs) combine the benefits of gelatin with the distinct characteristics of nanofibers, such as a high surface area-to-volume ratio and versatility, with an average diameter of 234 nm. This study describes their

exceptional reusability after multiple washing cycles, emphasizing their potential for environmental remediation. When gelatin nanofibers are coated with organophosphate-degrading enzymes such as Nus-OPH, OP compounds can be degraded directly on contaminated surfaces, such as farmers' clothes during the spraying of pesticides in the fields. This approach ensures real-time prevention of OP exposure, making it a feasible alternative for reducing environmental and occupational dangers. Gelatin nanofiber cross-linked with Nus-OPH enzyme is designed to reduce toxicity while increasing enzymatic activity, providing an effective approach for easing the health impacts of OP poisoning.

Organophosphate (OP) compounds are widely used in agriculture as pesticides and in industry as chemical agents, but their toxicity poses serious risks to human health and the environment. OPs inhibit acetylcholinesterase, an enzyme crucial for nervous system function, leading to severe poisoning symptoms such as muscle weakness, respiratory distress, and, in extreme cases, fatal outcomes. Exposure to these chemicals can occur accidentally or intentionally, making their detection and detoxification a priority for researchers. Novel approaches to OP mitigation involve enzyme immobilization on textiles and biodegradable nanocarriers that facilitate both degradation and treatment.

One promising strategy is the immobilization of enzymes capable of degrading OP compounds onto ready-made textiles. This method allows for real-time OP detection and breakdown, ensuring protection for individuals at risk of exposure, such as farmers, researchers, and military personnel. Unlike cross-linking techniques, immobilization maintains enzyme stability and effectiveness over time, making it a viable solution for wearable safety gear. By integrating enzyme-coated textiles into protective clothing, users can neutralize OP compounds upon contact, significantly reducing their toxic impact.

Moreover, the potential of biodegradable sodium alginate nanoparticles have been explored to enhance enzyme stability and provide a method for treating OP poisoning. These nanoparticles encapsulate the enzyme, ensuring its activity for extended periods and allowing for intravenous administration in medical applications. When delivered into the bloodstream, encapsulated enzymes break down OP molecules, preventing them from interfering with acetylcholinesterase and reducing toxicity. This method offers a promising therapeutic approach for OP poisoning cases, improving treatment efficacy and recovery outcomes.

Enzymes such as Organophosphorus Hydrolase (OPH) play a crucial role in OP degradation. OPH works by hydrolyzing OP compounds, rendering them less toxic. Research indicates that immobilizing OPH on nanocarriers improves stability and reusability, increasing its efficiency in OP detoxification. Nanocarriers provide a larger surface area for enzyme attachment, enhancing degradation speed and reliability (Cheng et al., 2018). These advancements support environmental cleanup efforts by reducing OP contamination in soil and water sources.

In addition to enzymatic detoxification, proteins have been found to bind to OP compounds, preventing them from inhibiting acetylcholinesterase. This mechanism presents an alternative strategy for OP poisoning treatment, as specialized antibodies can neutralize OP molecules before they cause neurological damage (Thakur et al., 2019). By incorporating these biological defense mechanisms into medical treatments, researchers can improve intervention methods for OP poisoning victims.

While enzyme immobilization and nanocarrier technology present promising solutions, challenges remain in optimizing enzyme stability, efficiency, and cost-effectiveness. Future research will focus on refining these techniques and gaining regulatory approval for widespread use. Advances in biotechnology and nanoscience will likely improve enzyme formulation and delivery methods, making OP degradation more accessible and effective. Through continued innovation and collaboration, the aim is to develop safer and more efficient ways to neutralize OP compounds, ultimately improving environmental and human health protection.

Chapter 2

Objectives

1. Functionalized Enzyme Loaded Textile for OP degradation

(A) Electrospun Nanofibre

(B) Readymade Textile

2. Enzyme Loaded Nanoparticle for OP Detoxification

3. Detection for OP Degradation Monitoring

Fig. 2.1. Diagrammatic representation shows a farmer wearing cloth immobilized with enzyme which can successfully degrade and prevent from OP induced toxicity.

Chapter 3

Materials and Methods

3.1. Materials

Strains: The *E. coli* Rosetta cells were used as host cells for expressing the enzyme Nus-OPH.

Chemicals: Luria Bertani Broth Miller (HiMedia), Kanamycin (HiMedia), Lysozyme, Tris-Cl, NaCl, Imidazole, Bis-Tris Propane buffer, CHES Buffer Ethyl Paraoxon (Sigma Aldrich), Coumaphos. BSA, Sodium Alginate, p-NP, IPTG, $MnCl_2$, $CoCl_2$, Glycine ($C_2H_5NO_2$), Ethylene Diamine Tetra Acetic Acid (EDTA), Sodium hydroxide (NaOH), Sodium dodecyl sulphate or Sodium lauryl sulphate (SDS), Coomassie brilliant blue G-250, β- mercaptoethanol ($HOCH_2CH_2SH$), Luria-Bertani broth (LB broth), Kanamycin, Acrylamide (C_3H_5NO), N, N′- Methylene bisacrylamide (MBAA), Triethylenetetramine or Tetramethyl ethylene diamine (TEMED), Ammonium per sulphate ($(NH_4)_2S_2O_8$), bromophenol blue or 3′,3″,5′,5″- tetra bromophenol sulfonphthalein ($C_{19}H_{10}Br4O_5S$), Bovine serum albumin (BSA), Isopropyl-β-D-thiogalactopyranoside ($C_9H_{18}O5S$), Imidazole ($C_3H_4N_2$), Glycerol ($C_3H_8O_3$), Methanol (CH_3OH), Glacial acetic acid (CH_3COOH), Ethanol (C_2H_5OH), 1,3 Bis [tris (hydroxymethyl) methylamino] propane (Bis-Tris propane) $C_{11}H_{26}N_2O_6$, 2- (Cyclohexyl amino) ethane sulfonic acid (CHES) ($C_8H_{17}NO_3S$), Ni NTA Sepharose beads, Glycine.

Dulbecco's Modified Eagle Medium (DMEM), Dimethyl Sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Fluorescein Isothiocyanate (FITC), Phosphate buffered saline (PBS), Trypsin.

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All chemicals were of molecular biology grade and procured from different manufacturers like Sigma Aldrich Chemical Pvt. Ltd., Invitrogen Pvt. Ltd., MP Biomedical USA, HiMedia Pvt. Ltd.India, Alfa Aesar Pvt. Ltd., Sisco Research Lab Pvt. Ltd. India, and Otto Chemie Pvt. Ltd.

3.2. Methods

3.2.1. Expression and purification of OPAA-FL

The primary culture was grown from a single colony of transformed cells which was inoculated into an LB broth tube with Kanamycin (10 μg/ml). The culture was incubated for 10-12 hours at 37 °C at 220 rpm. The required volume of culture was added from the primary culture to the secondary culture, and cells were kept at 37 °C on an incubator shaker till 0.8 units of OD was attained. The cells were induced with 1 mM IPTG and 1 mM $MnCl_2$ for the OPAA-FL variant. The culture was incubated at 180 rpm at 20 °C for 20 hours. After induction, the cells were harvested at 10,000 rpm at 4 °C for 10 minutes. The resultant pellet was washed and resuspended with 50 mM Tris-Cl (pH 8.0), 500 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 10% glycerol. To the lysed cells, lysozyme was added to a final concentration of 100 μg/ml. The cells were then incubated at 30 °C for 20 minutes. Lysozyme treatment was followed by sonication. The cell lysate was centrifuged at 14,000 rpm at 4 °C for 10 minutes. The lysate was then analyzed on 12% SDS-PAGE. The majority of expression was observed in the induced supernatant fraction.

The recombinant OPAA-FL variant protein was purified using a Ni-nitrilotriacetic acid affinity chromatography column at 4 °C since the OPAA-FL variant has an N-terminal His-Tag. The input sample was prepared in a way that it had the composition of the equilibration buffer (500 mM NaCl, 50 mM Tris-Cl, pH 8.0, and 10 mM Imidazole). The sample was loaded on a pre-equilibrated column at a flow rate of 1mL/min. The column was then washed with Wash buffer, which comprised of 50 mM Tris-Cl, pH 8.0, 500 mM NaCl and 50 mM Imidazole. The protein was eluted using an elution buffer containing 50 mM Tris-Cl, pH 8.0, 500 mM NaCl and 150 mM Imidazole. All fractions were analyzed on 12% SDS-PAGE.

3.2.2. Expression and purification of Nus-OPH enzyme

The primary culture was grown from a single colony of Nus-OPH transformed cells which was inoculated into an LB broth tube with Kanamycin (10 μg/ml). The culture was incubated for 10-12 hours at 37 °C at 220 rpm. The required volume of culture was added from the primary culture to the secondary culture, and cells were kept at 37 °C on an incubator shaker till 0.8 units of OD was attained. The cells were induced with 0.1 mM IPTG and 0.1 mM $CoCl_2$ for the Nus-OPH variant. The culture was incubated at 180 rpm at 16 °C for 16 hours. After induction, the cells were harvested at 10,000 rpm at 4 °C for 10 minutes. The resultant pellet was washed and resuspended with 50 mM Tris-Cl (pH 8.0),
500 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 10% glycerol. To the lysed cells, lysozyme was added to a final concentration of 100 μg/ml. The cells were then incubated at 30 °C for 20 minutes. Lysozyme treatment was followed by sonication. The cell lysate was centrifuged at 14,000 rpm at 4 °C for 10 minutes. The lysate was then analyzed on 12% SDS-PAGE. Most of the expression was observed in the induced supernatant fraction.

The recombinant Nus-OPH variant protein was purified using a Ni-nitrilotriacetic acid affinity chromatography column at 4 °C since the Nus-OPH variant has an N-terminal His- Tag. The input sample was prepared in a way that it had the composition of the equilibration buffer (500 mM NaCl, 50 mM Tris-Cl, pH 8.0, and 10 mM

Imidazole). The sample was loaded on a pre-equilibrated column at a flow rate of 1mL/min. The column was then washed with Wash buffer, which comprised of 50 mM Tris- Cl, pH 8.0, 500 mM NaCl and 50 mM Imidazole. The protein was eluted using an elution buffer containing 50 mM Tris-Cl, pH 8.0, 500 mM NaCl and 150 mM Imidazole. All fractions were analysed on 8% SDS-PAGE.

3.2.3. Bradford assay for determination of enzyme concentration

For standard assay, 5 µL of different concentrations of BSA (0 mg/mL to 1.2 mg/mL) were taken and 195 µL of bradford reagent was added in it. The samples were incubated in dark for 5 mins at room temperature. The absorbance was taken at 595 nm. Standard calibration curve was already prepared and unknown concentrations of protein were then analyzed using the standard calibration curve.

3.2.4. Activity assay of recombinant protein OPAA-FL and Nus-OPH

1 µl of pure Nus-OPH protein, pH 9.0, 0.1 mM $CoCl_2$, 50 mM CHES buffer with 1 mM ethyl paraoxon (Sigma) and

0.2 mm Coumaphos respectively were used to perform an activity assay for Nus- OPH. The final volume was made up to 200 µl with distilled water. For activity of coumaphos, 1% Triton X-100 was added in solution to enhance solubility of coumaphos. The preparation of a blank sample was similar, except no enzyme was added. For 5 minutes, the reaction mixtures were incubated at 37 °C. While for OPAA-FL activity, 1 µl of pure OPAA-FL protein, pH 8.5, 1 mM $MnCl_2$, 50 mM BTP buffer with 1 mM Ethyl Paraoxon (Sigma) was used to perform an activity assay for OPAA-FL. The preparation of a blank sample was similar, except no enzyme was added. For 15 minutes, the reaction mixtures were incubated at 50 °C.

By measuring the absorbance of p-nitrophenol generated by the hydrolysis of ethyl paraoxon at 410 nm (Extinction coefficient = 17,000/M/cm for pNP), the activity was estimated employing a plate reader (SYNERGY H1 microplate reader). For chloreferon, standard calibration curve of chloreferon was used. In milligrams of total protein, specific activities were expressed as units (micromoles of ethyl paraoxon hydrolyzed per minute) per mg. The degradation mechanism of ethyl paraoxon and coumaphos is depicted in **Fig. 3.1.** and **Fig. 3.2.** respectively.

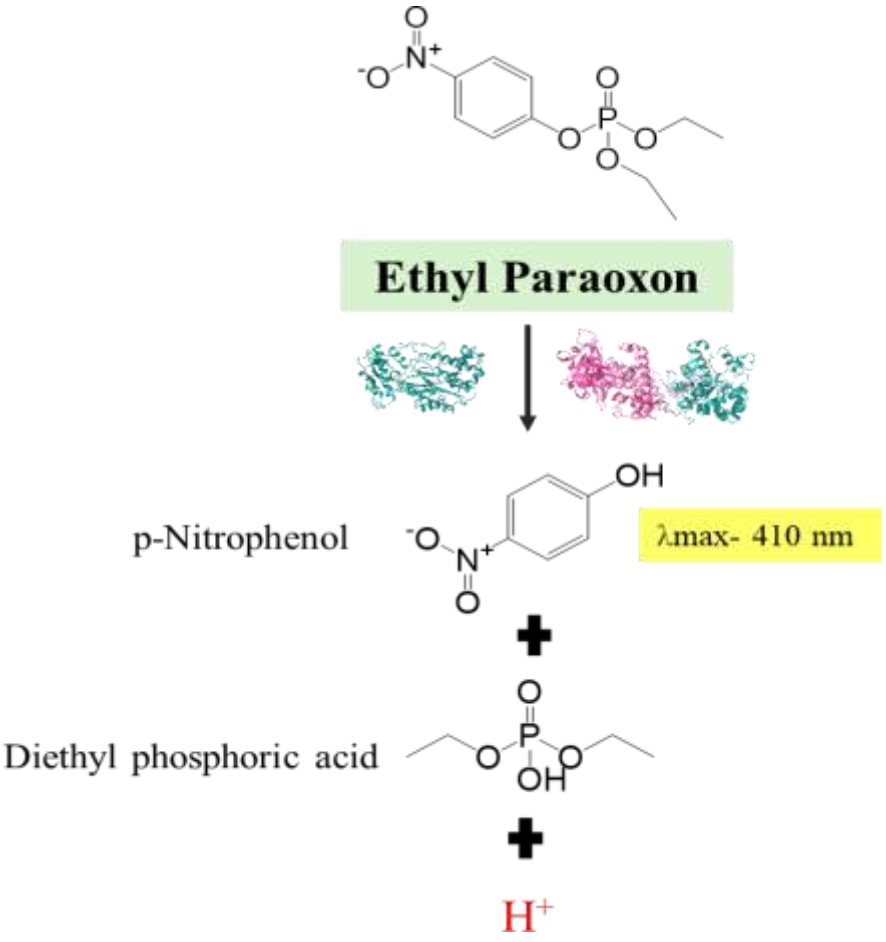


Fig. 3.1.: Mechanism of degradation of EP with recombinant OP hydrolyzing enzymes.

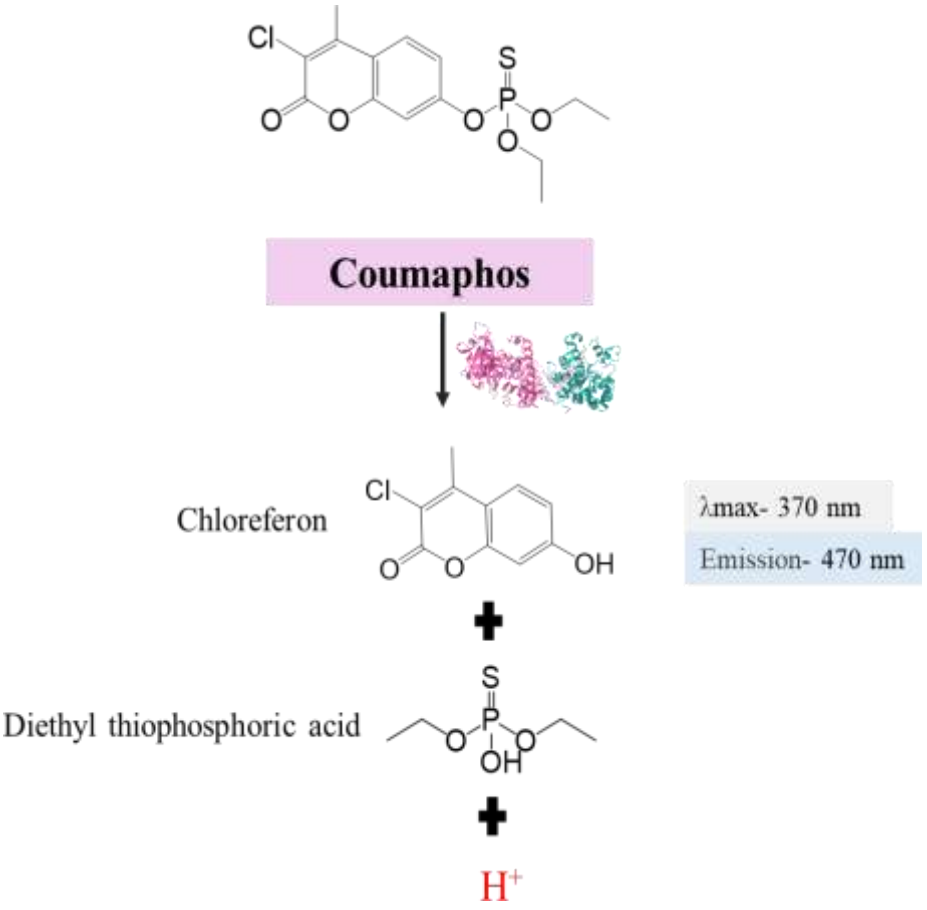


Fig. 3.2.: Mechanism of degradation of Coumaphos with recombinant OP hydrolyzing enzymes.

3.2.5. Preparation of electrospun nanofibers

The principle of electrospinning is that a high electric field is applied to stretch and elongate the charged polymer to make a nanofiber *via* taylor cone formation. A high electric field usually in kilo-volts is applied. A syringe is present to load the polymer onto which regulates the flow of the viscous liquid. The drop forms at the top if the syringe gets charged by an electric field and molds into a taylor cone. The polymer is collected on the collector drum which dried to form a nanofiber. The formed nanofiber is usually extracted from the aluminum foil and ready for conducting various experiments.

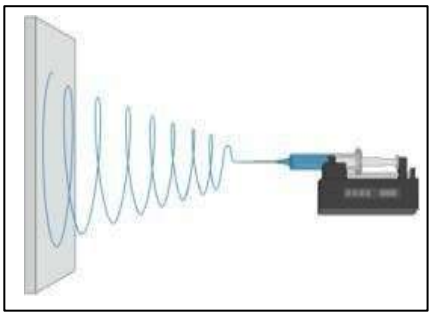
3.2.5.1. Type-A Gelatin (25 % w/v) Electrospun Nanofiber

For preparation of 25% w/v Type-A Gelatin electrospun nanofibers, 7.5 gm of Type-A Gelatin powder was dissolved in glacial acetic acid and water to make a final volume of 30 ml in the water to glacial acetic ratio of 1:9 (30 ml water and 27 ml glacial acetic acid) (Ratanavaraporn *et al*., 2010). Electrospinning was performed using following parameters followed by drying our sample at room temperature for overnight. **Table 3.1.** shows the electrospinning parameters used for electrospinning of gelatin nanofibers. Electrospinning unit showing taylor cone formation by the charged polymer to form a nanofiber is illustrated in **Fig. 3.3.**

Table 3.1.: Electrospinning parameters for preparation of Type-A Gelatin (25% w/v) Electrospun Nanofibers

Parameters	Values
Flow Rate	1.2 ml/min
Volume of sample	4 ml
Volume of syringe used	5 ml
Internal Diameter of Syringe	13.07 mm
Voltage	16 kV

(A) **(B)**



(C)

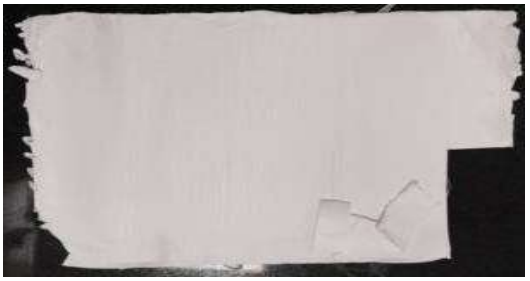


Fig. 3.3.: Electrospun Nanofiber **(A)** Electrospinning unit **(B)** Taylor Cone Formation **(C)** Electrospun Nanofibers directly taken out from the aluminum foil.

3.2.5.2. SEM characterization of Gelatin nanofibers

The characterization of nanofibers was done using Scanning Electron Microscopy (SEM). It is a crucial step in understanding its morphology and structure. SEM provides high-resolution images that reveal the surface morphology, fiber diameter, and pore size of the nanofibers. SEM uses a high-energy beam of focused electrons to produce detailed images of material surfaces. When the electrons interact with the sample's surface, generation of various signals occurs, such as secondary electrons, backscattered electrons, along with characteristic X-rays. These signals are captured by detectors which then form an image that provides insights into the sample‘s topography, composition, and other properties.

3.2.5.3. Cross-linking gelatin nanofibers *via* glutaraldehyde

After the formation of gelatin nanofiber, for the purpose of cross-linking the fiber has been put into the desiccator for 12 hours having 25 % glutaraldehyde and due to the vapor formation cross-linking happens as depicted in **Fig. 3.4.** The reason behind the cross-linking is that the aldehyde groups of glutaraldehyde react with the amine groups on the gelatin, forming imine bonds. These imine bonds can further react with other amine groups, creating cross-links. Glutaraldehyde treated fabric cut into 2×2 cm pieces with a average weight of 61.9 mg ± 7.3 mg. Crosslinking efficiency was evaluated by estimating the relative activity of unwashed fabric with initial washed fabric.

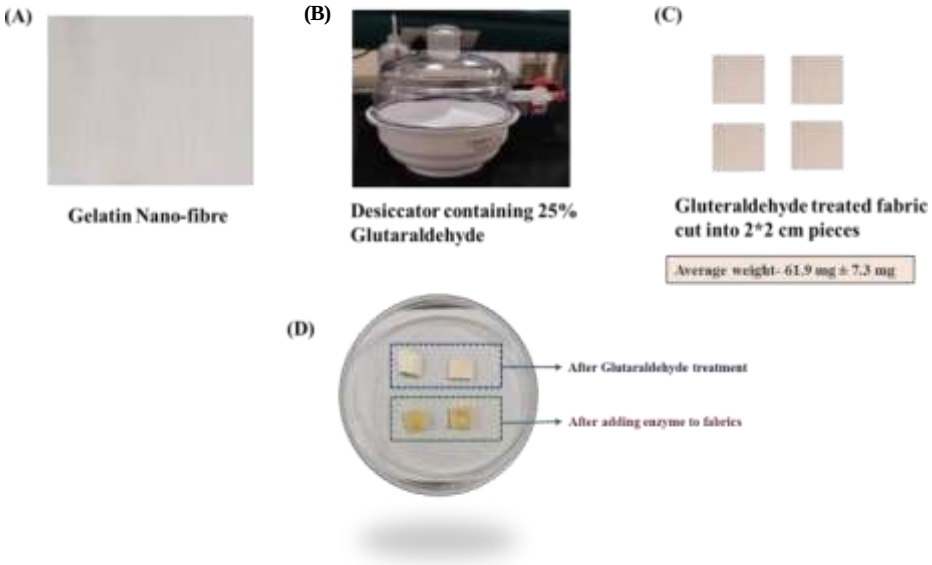


Fig. 3.4.: Cross-linking via glutaraldehyde **(A)** Gelatin Nanofiber (B) Desiccator containing 25 % glutaraldehyde (C) After 12 hours, glutaraldehyde treated fabric cut into 2*2 cm pieces (D) Petri Plate containing nanofibers after glutaraldehyde treatment and nanofibers after glutaraldehyde treatment and adding enzyme to fabrics.

3.2.5.4. Swelling assay of gelatin nanofiber

The swelling assay of gelatin nanofibers is performed to evaluate their water absorption capacity and retention ability. This is an important step because it helps assess the fibers' structural and functional properties, especially in applications where they interact with fluids. By understanding how much liquid the nanofibers can absorb and retain, we can determine their suitability for specific applications and how well they mimic biological environments or meet practical requirements. To conduct the swelling assay of gelatin nanofibers, we first carefully weighed the fibers to record their initial weight. After this, we placed the fibers in a buffer solution and allowed them to swell for a duration of 1 hour. Once the swelling period was complete, we measured the fibers' retention capacity to assess how much liquid they had absorbed during this time.

3.2.5.5. Bioremediation of Ethyl Paraoxon using Nus- OPH loaded gelatin nanofibers

To analyze the depletion of the substrate Ethyl Paraoxon (EP) using a SYNERGY H1 microplate reader by measuring the absorbance of para-nitrophenol (PNP) at 410 nm, which forms during EP degradation. However, the presence of other compounds, such as glutaraldehyde, gelatin nanofibers itself, and acetic acid, interferes with the absorbance readings and makes the analysis less reliable. In contrast, High-Performance Liquid Chromatography (HPLC) provides a clear advantage by separating individual compounds and giving distinct peaks for each, which eliminates confusion and improves the accuracy of the results. Using HPLC, we generated a standard calibration curve for EP, which allowed us to quantify the degradation of EP and the formation of PNP with greater precision.

3.2.5.6. Bioremediation of CM using Nus- OPH loaded Gelatin nanofibers

To analyze the depletion of the substrate Coumaphos using a SYNERGY H1 microplate reader by measuring the absorbance of fluorescent compound, Chloreferon at 370 nm, which forms during Coumaphos degradation. However, the presence of other compounds, such as glutaraldehyde, gelatin nanofibers itself, and acetic acid, interferes with the absorbance readings and makes the analysis less reliable. In contrast, High-Performance Liquid Chromatography (HPLC) provides a clear advantage by separating individual compounds and giving distinct peaks for each, which eliminates confusion and improves the accuracy of the results. Using HPLC, we generated a standard calibration curve for Chloreferon, which allowed us to quantify the degradation of Coumaphos with greater precision.

3.2.5.7. Reusability of Enzyme Linked Nanofibers and readymade cotton textile

The enzyme assay carried out using Nus-OPH loaded enzyme fabric/fibre. Conditions were 1mM EP, 0.1 mM $CoCl_2$ for 10 mins at 37 °C, measured the absorbance at 410 nm and then washed it with 10 ml of D/W. We carried multiple cycle of enzyme assay. The relative activity was evaluated by normalizing the initial wash activity of gelatin nanofiber to be 100%.

3.2.5.8. Detection of Ethyl Paraoxon using Nus-OPH loaded Gelatin nanofiber

Gelatin nanofibers are prepared and cross-linked with the Nus-OPH enzyme in a 25% glutaraldehyde solution. Following the cross-linking process, an enzyme of 0.03 U is adsorbed to the nanofibers. These fibers are then allowed to dry, first at ambient temperature and later at 4°C. A reaction mixture is made with 50 mM CHES buffer, and 0.1 mM $CoCl_2$ with various concentrations of EP and was incubated at 37 °C for 10 minutes. The absorbance was measured at 410 nm by Synergy H1 microplate reader.

3.2.5.9. Detection of Caumophos using Nus-OPH loaded Gelatin nanofiber

Gelatin nanofibers are prepared and cross-linked with the Nus-OPH enzyme in a 25% glutaraldehyde solution. Following the cross-linking process, an enzyme of 0.03 U is administered to the nanofibers. These fibers are then allowed to dry, first at ambient temperature and later at 4°C. A reaction mixture is made with 50 mM CHES buffer, and 0.1 mM $CoCl_2$ with various concentrations of Coumaphos and was incubated at 37 °C for 10 minutes. The absorbance was measured at 410 nm by Synergy H1 microplate reader.

3.2.6. Degradation of OP compounds from readymade cotton clothes

Autoclaved cotton clothes were cut in squares with dimension of 2×2 cm. A known concentration of enzymes (Nus-OPH and OPAA-FL) was added to the fabric materials and air-dried at room temperature for 4-5 hours for complete absorption of enzyme onto fabric material as shown in **Fig. 3.5**. OPAA-FL enzyme loaded cotton fabrics were incubated with 100 ppm of EP with 50 mM CHES buffer (pH-9.0), 1 mM $CoCl_2$ at 50 °C for 180 minutes. While Nus-OPH enzyme loaded cotton fabrics were then incubated with 100 ppm of OP substrates (EP and Caumophos) with 50 mM CHES buffer (pH 9.0), 1 mM $CoCl_2$ at 37 °C for 180 minutes. For Caumophos degradation, additional 1% Triton X-100 was added to enhance solubility of Caumophos. A blank sample (cloth with no enzyme) was also incubated with OP compounds to monitor self-hydrolysis. Additionally, to imitate real life scenario, similar experiment with all other reagents except buffer was conducted with tap water in place of buffer. For EP degradation, absorbance of p-nitrophenol was taken at 410 nm while for Caumophos degradation, absorbance of chloreferon was analyzed at 370 nm using SYNERGY H1 Microtek plate reader. % Degradation efficiency was calculated for tap and buffer conditions for OPAA-FL and Nus-OPH enzyme loaded fabric.

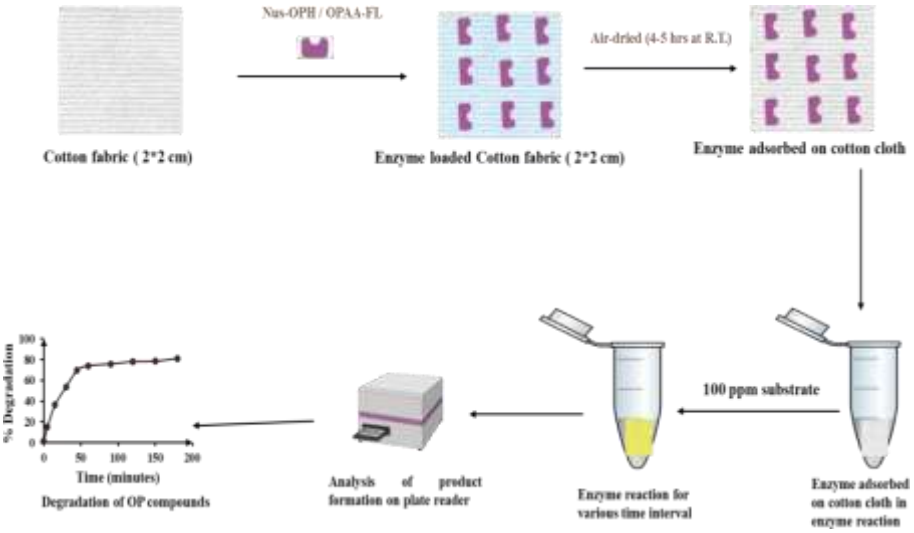


Fig. 3.5.: Mechanism of degradation of EP and Caumophos with recombinant OP hydrolyzing enzymes loaded on cotton clothes.

3.2.7. Detection of Ethyl Paraoxon using Nus-OPH loaded cotton cloth by RGB analysis

The principle of RGB analysis is based on the three primary colors: Red, Green, and Blue. These colors combine in varying proportions to produce a wide range of other colors. In RGB analysis, each color is represented by a specific value, indicating its intensity in the overall color mixture. When analyzing a sample, the colors observed are influenced by how much of each primary color is reflected or absorbed. If a particular color trend appears dominant in the analysis, it means that the opposite color (as seen on a color wheel) is being absorbed by the sample as seen in **Fig. 3.6.** To carry out the experiment, we began by cutting the cloth into small pieces of a specific size, in this case, measuring 1× 1 cm. After that, we applied the Nus-OPH enzyme onto the surface of the cloth pieces. Once the enzyme was added, we left the cloth to dry naturally at the room temperature. We prepared a reaction mixture that contained different concentrations of EP (the compound we wanted to detect). Once the reaction mixture was ready, we carefully added a measured amount of it onto each piece of cloth. The cloth pieces were then placed in an

incubator at a temperature of 37 °C for 10 minutes to allow the reaction to take place. This step ensured that the enzyme and the EP could interact with each other.

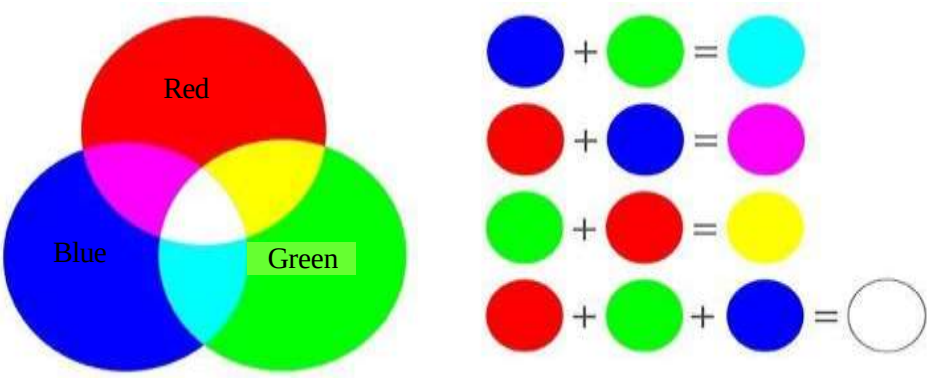


Fig. 3.6.: Pictorial representation of three primary colors used in RGB analysis.

3.2.8. Ethyl Paraoxon degradation on cotton textile fabric by Nus-OPH

An experiment is performed to demonstrate the breakdown of organophosphate (OP) compounds with the enzyme Nus- OPH. The enzyme was applied to a fabric, air dried, and stored at 4 degrees. To assess its efficiency, the enzyme- coated fabric was tested for 180 minutes under two conditions: tap water and CHES buffer. In both settings, a reaction mixture containing the OP chemical was injected, and the degradation rate was measured over time. The appearance of a yellow tint, which indicates para- nitrophenol (PNP) synthesis, confirmed the breakdown of OP compounds.

3.2.9. Caumophos degradation on cotton textile fabric by Nus-OPH

The degradation of organophosphate (OP) compounds was demonstrated with the enzyme Nus-OPH. The enzyme was applied to a fabric, air dried, and stored at 4°C. To assess its efficiency, the enzyme-coated fabric was tested for 180 minutes under two conditions: tap water and CHES buffer. In both settings, a reaction mixture containing the OP chemical was injected, and the degradation rate was measured over time. The fluorescence intensity is measured of Chloreferon, excitation at 370 and emission at 470 nm, confirmed the breakdown of OP compounds.

3.2.10. Ethyl Paraoxon degradation on textile by OPAA- FL

The degradation of organophosphate (OP) compounds was demonstrated with the enzyme OPAA-FL. The enzyme was applied to a fabric, air dried, and stored at 4°C. To assess its efficiency, the enzyme-coated fabric was tested for 180 minutes under two conditions: tap water and BTP buffer. In both settings, a reaction mixture containing the OP chemical was injected, and the degradation rate was measured over time. The appearance of a yellow tint, which indicates para- nitrophenol (PNP) synthesis, confirmed the breakdown of OP compounds.

3.2.11. Detection of Ethyl Paraoxon with varied concentrations using Nus-OPH loaded cotton cloth (Solution Phase)

This experiment included varied EP concentrations ranging from 0 to 80 ppm. Prior to the reaction, the enzyme is applied to the cloth, which is air-dried and stored at 4 °C. A reaction mixture is made with varying EP concentrations and incubated for 10 minutes. The absorbance of para- nitrophenol (PNP), the breakdown product, is then measured at 400 nm to validate the enzyme's activity.

3.2.12. Reusability of readymade cotton textile

The approach was to involve immobilizing the enzyme Nus- OPH onto ready-made textiles and determining its reusability by measuring how long it remains attached after numerous washes. The enzyme is absorbed onto the cloth, air-dried, and stored at 4 degrees temperature. To assess its activity, a reaction mixture is created and monitored for the appearance of yellow color, which indicates the formation of para-nitrophenol. This yellow color confirms the degradation of EP into PNP, demonstrating the enzyme's efficacy on the textile. This method offers a practical and effective option for assessing pesticide degradation and reusability in fabrics.

3.2.13. Preparation of sodium alginate nanoparticles

A stock solution of 0.6 % sodium alginate solution is prepared in 10 ml of water. On the other hand, 4% calcium chloride solution is made in 40 ml of water for cross-linking. 1 ml of the sodium alginate solution is mixed thoroughly with 1 ml of purified OPH enzyme. This will result in a final sodium alginate concentration of 0.3% when combined with the enzyme. Clean the ultrasonic atomizer using first acetone and then water. Next, load the prepared mixture into a syringe. The solution is then processed using specific parameters. Suspended nanoparticles in the calcium chloride solution, separated by centrifuging at 5,000 rpm for 15 minutes as shown in **Fig. 3.7**.

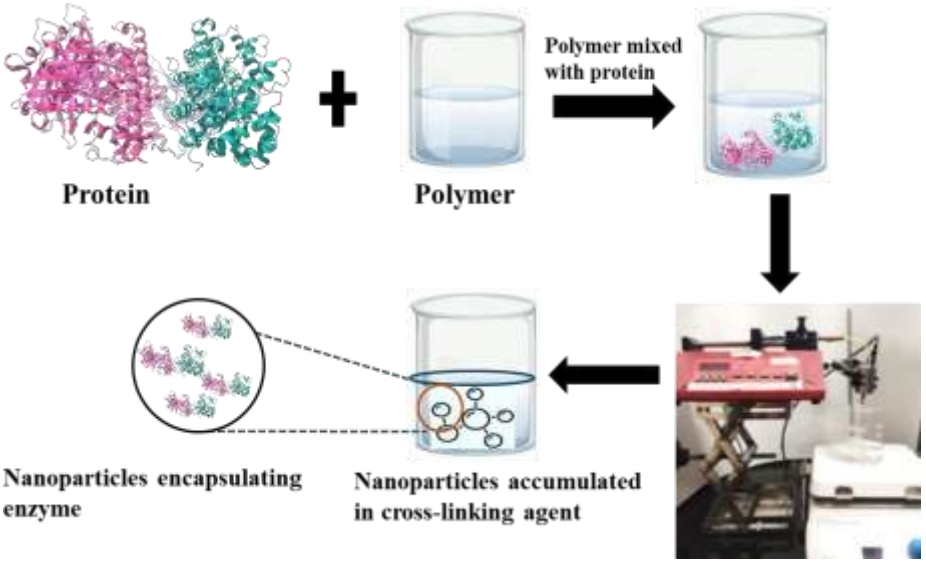


Fig. 3.7.: Diagrammatic representation of enzyme loaded nanoparticle preparation using ultrasonic atomizer.

Wash the pellet three times to remove any unbound protein. Finally, re-suspend the pellet in 1 ml of distilled water and store it at 4 °C (Kumar *et al*., 2014). Nus-OPH loaded sodium alginate nanoparticles, showing the formation of PNP after EP degradation as shown in **Fig. 3.8.**

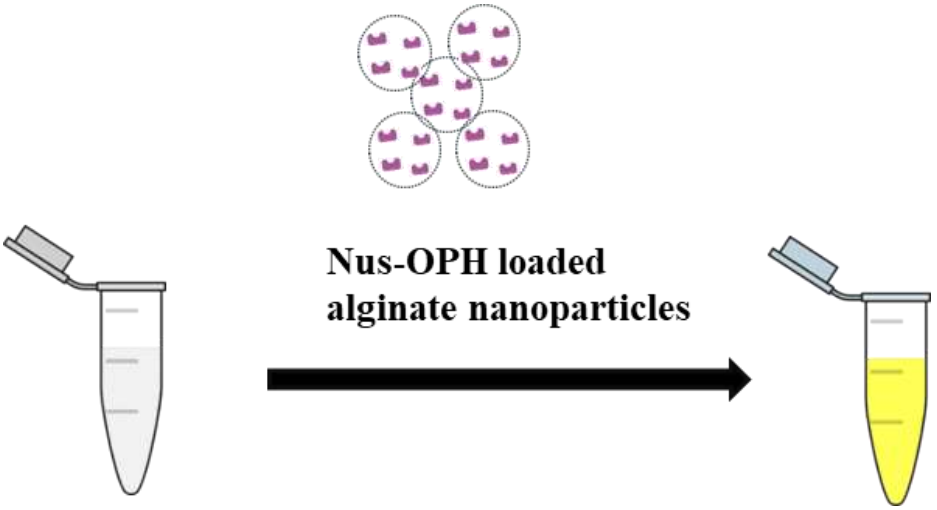


Fig. 3.8.: Schematic representation of degradation of EP into PNP (yellow color) using Nus-OPH loaded alginate nanoparticles.

The characterization of Nus-OPH loaded nanoparticles was further done using SEM and DLS. Also, enzyme efficiency was evaluated by conducting enzyme assay using EP and Coumaphos as substrate. The parameters followed during the synthesis of 0.3% Nus-OPH- Sodium alginate nanoparticles is shown in the **Table 3.2.**

Table 3.2.: Nanoparticle synthesis parameters for preparation of 0.3% Nus-OPH- Sodium alginate nanoparticles

Parameters	**Values**
Flow Rate	0.2 ml/min.
Volume of sample	5 ml
Internal Diameter	12 mm
Voltage	4.2 watts
Frequency	130 kHz

3.2.14. MTT cell viability assay

The MTT assay is a widely used method to assess cell viability by measuring the metabolic activity of living cells. It involves the use of a yellow compound called MTT, which is converted into insoluble purple formazan crystals by mitochondrial enzymes present in active cells. The amount of formazan produced reflects the number of metabolically active cells, as non-viable cells cannot perform this conversion. After the reaction, the formazan crystals are dissolved in DMSO, and the absorbance of the solution is measured. We tested how different substances affect the survival of kidney cells (HEK-293). We looked at Ethyl Paraoxon (EP), plain sodium alginate nanoparticles, and sodium alginate nanoparticles with Nus- OPH enzyme as shown in **Fig. 3.9.** To measure the effect, MTT assay was performed. Firstly, the 10,000 healthy kidney cells were placed into each section of a 96-well plate and let them settle down.

Then, the exposure was given to cells by either the pesticide (EP) or the nanoparticles and let them incubate for 48 hours. After the incubation period, we added a special chemical (MTT solution), which reacts with living cells, turning from yellow to purple. This took about three hours. Then, DMSO was added to dissolve the purple crystals, making it easier to measure the color intensity. Using a microplate reader, we checked the absorbance at 595 nm.

Finally, the survived cells were calculated by comparing the results to a control sample (untreated cells). This gave us the percentage of cell viability for each substances at a specific wavelength (550 nm) using a plate reader.

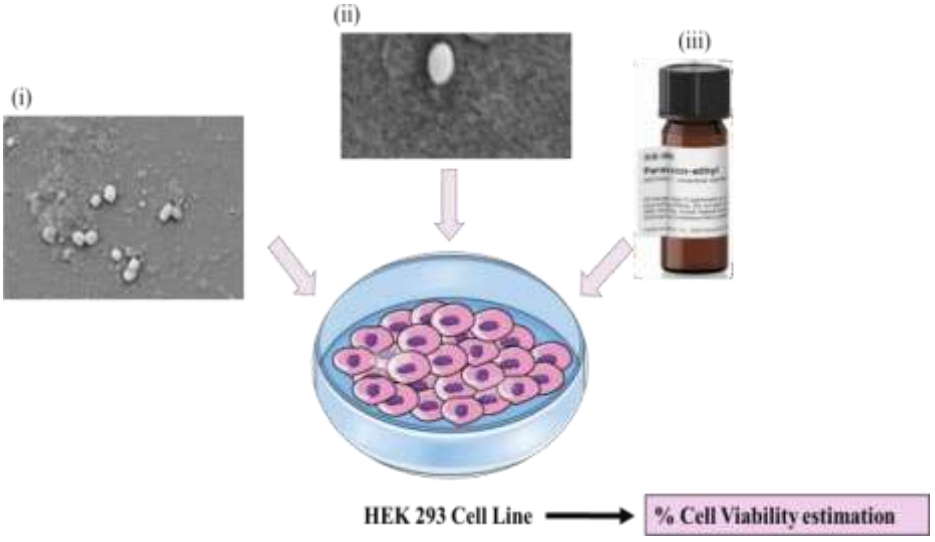


Fig. 3.9.: Schematic representation of % cell viability assay. MTT of **(i)** Sodium Alginate Nanoparticles encapsulated Nus- OPH enzyme. **(ii)** Bare Sodium Alginate Nanoparticles. **(iii)** Ethyl Paraoxon has been done

3.2.15. Cellular uptake of sodium alginate nanoparticles

The purified Nus-OPH protein was obtained and mixed with sodium alginate in equal amounts. The mixture was stirred well, and sodium alginate nanoparticles were formed to encapsulate the enzyme, as described in **3.2.13.** FITC (0.1 mg/mL) was then added to the nanoparticles and left for 8 hours for tagging. After that, the nanoparticles were washed with water three times to remove untagged

FITC and reduce background noise. HEK-293 cells were treated with these tagged nanoparticles, and the results were observed after 6 hours. A Diagrammatic representation showing the uptake of sodium alginate nanoparticles by HEK-293 cell line is shown in **Fig. 3.10**.

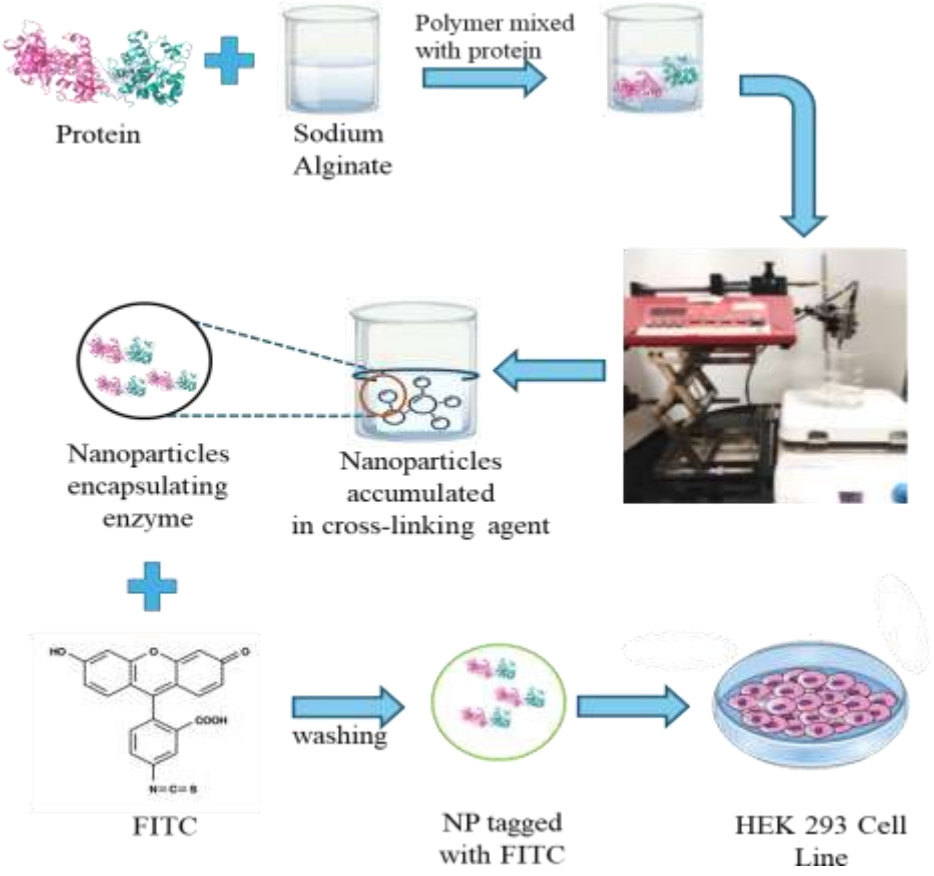


Fig. 3.10.: Diagrammatic representation showing the uptake of sodium alginate nanoparticles by HEK-293 cell line.

Chapter-4

Functionalized Enzyme Loaded Electrospun Gelatin Nanofibre for OP Compound degradation

4.1. Introduction

Pesticides are chemicals used to control pests, weeds, and plant infections. With expanding urbanization, the agriculture industry under enormous strain, resulting in increased pesticide use. This increased reliance is a major source of concern because of the negative impact on the environment and the serious health hazards it poses. (Aktar et al., 2009) Several organophosphate (OP) hydrolyzing enzymes have been discovered from various animals, including mammals; however, the most efficient and potent ones are found in bacteria. Organophosphorus hydrolase (OPH), organophosphorus acid hydrolase (OPAA), phosphotriesterase-like lactonases (PLL), methyl parathion hydrolase (MPH), and SsoPox are some examples of microbial OP hydrolyzing enzymes (Thakur et al., 2019).

In this study, the recombinant enzymes Nus-OPH and OPAA-FL were expressed in *E. coli* Rosetta (DE3), purified, and tested for their capacity to degrade OP pesticides. Coumaphos, an organophosphate pesticide commonly used in agriculture to control pests such as insects, mites, and ticks, was found to be degradable by these enzymes. While effective against pests on crops such as corn and fruit trees, Coumaphos is extremely harmful to humans and the environment. The US Environmental Protection Agency classifies it as a Group D carcinogen and has been related to significant health consequences such as respiratory difficulties and neurological disorders. The kinetic characteristics of Coumaphos degradation by OPAA- FL were further investigated.

The study also looked at another organophosphate, Ethyl Paraoxon. As an acetylcholinesterase inhibitor, it inhibits nerve

signal transmission and is highly harmful to humans and the environment.

4.2. Results and discussions

4.2.1. Expression and purification of OPAA-FL

Recombinant OPAA-FL was expressed in *E. coli* Rosetta (DE3) containing recombinant p28a- OPAA-FL vector using 1mM IPTG and 1 mM $MnCl_2$. **Fig. 4.1.** shows the results of OPAA- FL expression in both the pellet and supernatant fractions. The induced supernatant showed higher level of expression in induced supernatant fraction when compared to uninduced fractions and induced pellet. The recombinant OPAA-FL protein was purified using the metal affinity chromatography using Ni-NTA resin. The induction and purification were verified on a 12% SDS- PAGE gel. The protein was eluted in 150 mM Imidazole fraction (lane 8). The protein was dialyzed in 50 mM Tris and 50 mM NaCl and used for further assay.

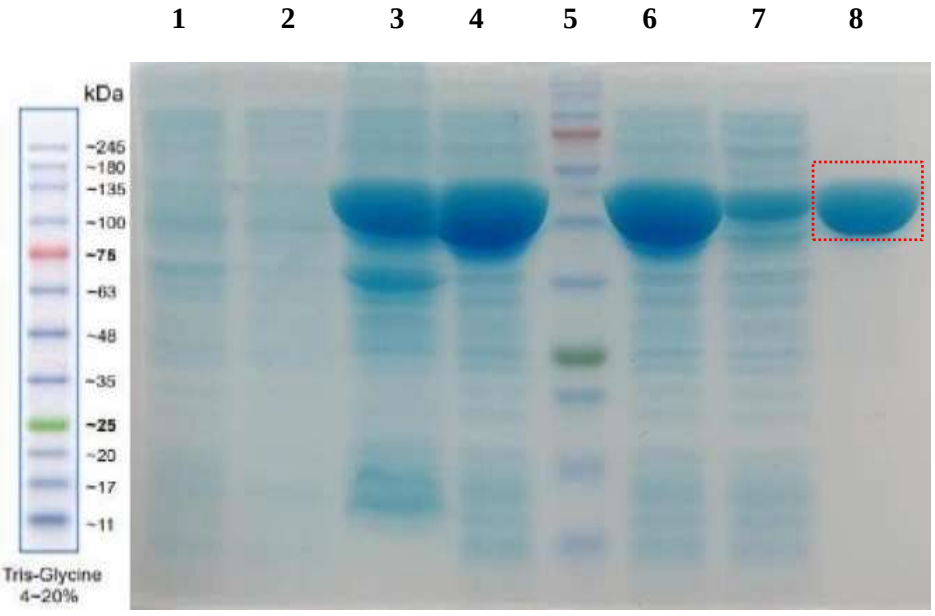


Fig. 4.1.: Expression and purification of OPAA-FL protein. Lane 1: Uninduced pellet; Lane 2: Uninduced supernatant; Lane 3: Induced pellet; Lane 4: Induced Supernatant; Lane 5: Ladder; Lane 6: Input; Lane 7: Flowthrough; Lane 8: Purified sample **(50 kDa)**

4.2.2. Expression and purification of Nus-OPH

Recombinant Nus-OPH was expressed in *E. coli* Rosetta (DE3) containing recombinant p28- Nus-OPH vector using 0.1 mM IPTG and 0.1 mM $CoCl_2$. **Fig.4.2.** shows the results of Nus- OPH expression in both the uninduced pellet and supernatant and induced pellet and supernatant fractions. The induced supernatant showed higher level of expression in induced supernatant fraction when compared to uninduced fractions and induced pellet. The recombinant Nus-OPH protein was purified using the metal affinity chromatography using Ni-NTA resin. The induction and purification were verified on an 8% SDS- PAGE gel. The protein was eluted in 150 mM Imidazole fraction (lane 8). The protein was dialyzed in 50 mM Tris and 500 mM NaCl and used for further assay.

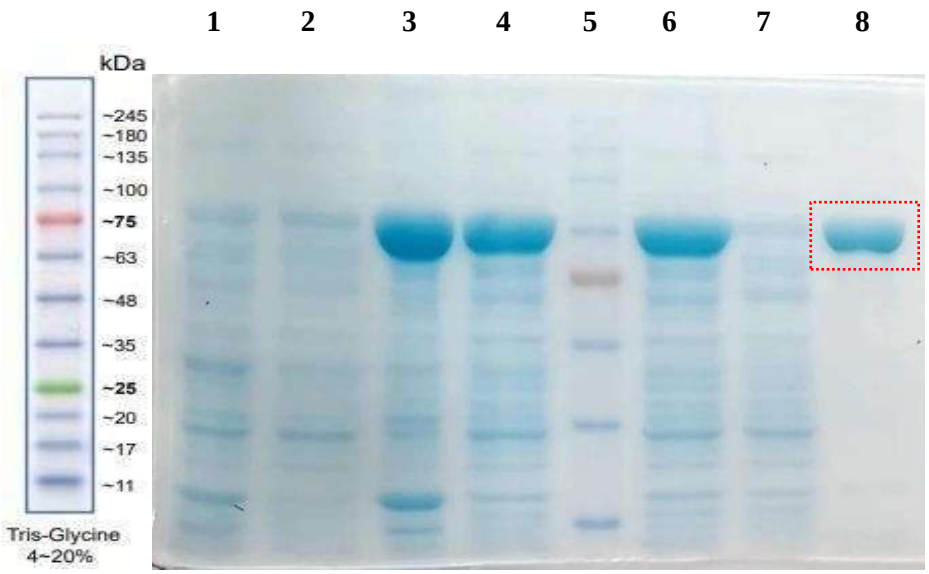


Fig. 4.2.: Expression and purification of Nus-OPH protein. Lane 1: Uninduced pellet; Lane 2: Uninduced supernatant; Lane 3: Induced pellet; Lane 4: Induced Supernatant; Lane 5: Ladder; Lane 6: Input; Lane 7: Flowthrough; Lane 8: Purified sample **(93 kDa)**

4.2.3. Bradford assay for determination of enzyme concentration

Standard calibration curve for BSA was prepared using bradford assay with linearity of 0.9948. The equation for bradford assay was obtained as y = 0.7388x + 0.0079. The concentration of OPAA-FL was obtained to be 10 mg/mL and Nus-OPH was obtained to be 0.5 mg/mL.

4.2.4. Activity assay of recombinant protein OPAA-FL and Nus-OPH

The purified enzymes were then checked for its " Specific Activity" which is defined as the micromoles of substrate hydrolyzed in one minute per mg of protein. In case of the degradation of 1 mM EP the specific activity came around to be 4.5 U/mg as shown in **Fig. 4.3.**

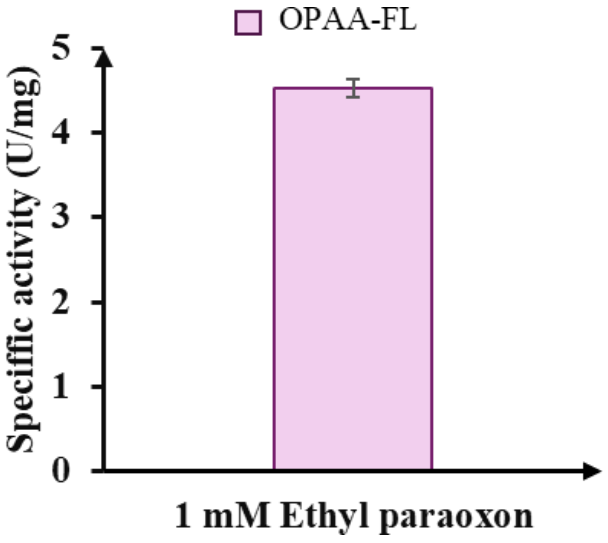


Fig. 4.3.: Degradation of EP by OPAA-FL with specific activity of 4.5 U/mg.

While in case of Nus-OPH, the specific activity came around to be 55 U/mg in 1 mM EP degradation. This suggestes that Nus-OPH can efficiently degrade the EP into its byproducts as depicted in **Fig. 4.4.**

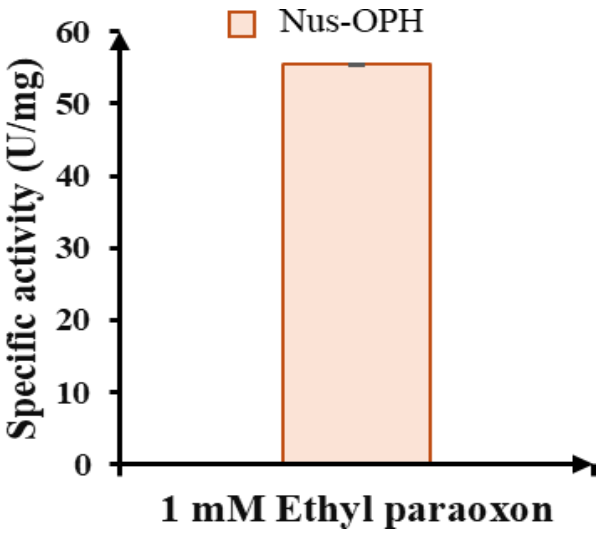


Fig. 4.4.: Degradation of EP by Nus-OPH with specific activity of 55 U/mg.

The specific activity of Nus-OPH in degrading 0.2 mM was around 0.02 U/mg as shown in **Fig. 4.5.**

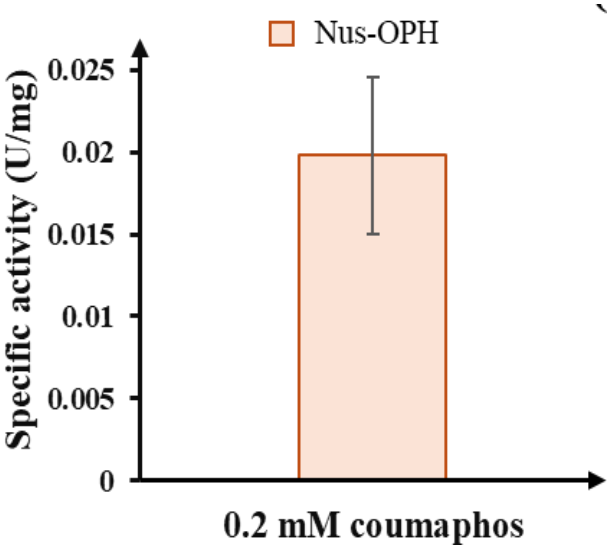


Fig. 4.5.: Degradation of EP by Nus-OPH with specific activity of 0.02 U/mg.

4.3. Summary

Enzymatic degradation of pesticides is a viable alternative for bioremediation of polluted soils and waters. Enzymes effectively degrade chemical bonds in pesticides, converting them into less harmful and biodegradable molecules. Organophosphate-degrading enzymes, such as Nus-OPH and OPAA-FL, can hydrolyze ester bonds in organophosphate compounds and convert them to innocuous chemicals. Nus- OPH shows high efficiency in degrading EP with 55 U/mg of specific activity, making Nus-OPH a n efficient enzyme to degrade EP. This technology has major advantages over traditional remediation approaches, including cost- effectiveness, environmental friendliness, and minimum disruption to ecosystems. Enzymatic degradation sets the path for long-term pesticide-reduction methods that safeguard both the environment and human health.

Chapter 5

Fuctionalised enzyme loaded Gelatin nanofibres application in Organophosphate Compound degradation

5.1. Introduction

Electrospun nanofibers have emerged as a strong powerful tool for pesticide degradation due to their outstanding structural and functional features, making them ideal for environmental remediation. (Kishimoto et al., 2022) These nanofibers have a high surface area-to-volume ratio, which allows for efficient interactions with pesticides, resulting in accelerated breakdown (Colín-Orozco et al., 2024). Furthermore, their porous nature enables controlled pesticide diffusion to active areas while limiting surface blockage. Electrospun nanofibers can be easily functionalized with active agents such as OPH enzymes, which increase their activity and stability during pesticide degradation (Nanlohy et al., 2025). Their durability and reusability make them an excellent choice for applications that require many cycles under varied environmental conditions. Furthermore, many of these nanofibers are made from biodegradable or recyclable polymers, which contribute to ecologically friendly solutions (Scaffaro et al., 2024).

In this study, electrospun nanofibers made of 25% w/v gelatin cross-linked with glutaraldehyde were successfully used to detect and disintegrate pesticides, specifically EP and CM. Detection was done in the solution phase with the H1 SYNERGY plate reader and sophisticated High Performance Liquid Chromatography (HPLC) methods. The reusability of these nanofibers was demonstrated by measuring pesticide degradation over numerous cycles. Furthermore, the study investigated the synthesis of heteropolymer electrospun nanofibers, which were effectively produced utilizing a PVA/PVP polymer combination, broadening the range of applications.

This study's findings show that electrospun nanofibers, particularly gelatin-based and heteropolymer nanofibers, can be highly efficient, long-lasting, and ecologically harmless materials for pesticide degradation and detection. This study demonstrates the unique use of sophisticated materials and analytical methodologies, paving the door for long-term solutions in environmental remediation and agricultural safety. This study provides significant advances for tackling pesticide-related difficulties by demonstrating the effective application and reusability of enzyme-functionalized nanofibers. These flexible materials considerably improve detection and degrading efficiency while also encouraging sustainable agricultural and environmental activities.

5.2. Results and Discussions

5.2.1. Type-A Gelatin (25% w/v) Electrospun nanofiber

Type-A Gelatin nanofibers were prepared *via* electrospinning using 25 % gelatin dissolved in 9:1 acetic acid: water. Around 15 mL of sample was used for preparation of nanofibers using flow rate of 1.2 mL/hour. The nanofiber was initially evaluated using inverted microscope. **Fig. 5.1(A)** shows 20X magnification of gelatin nanofibers when evaluated using inverted microscope. To give a clear distinction between glass slide and electrospun nanofiber, an image was taken which is represented in **Fig. 5.1(B)**. **Fig. 5.1 (C)** shows the detached gelatin nanofiber from aluminum sheet after nanofiber formation.

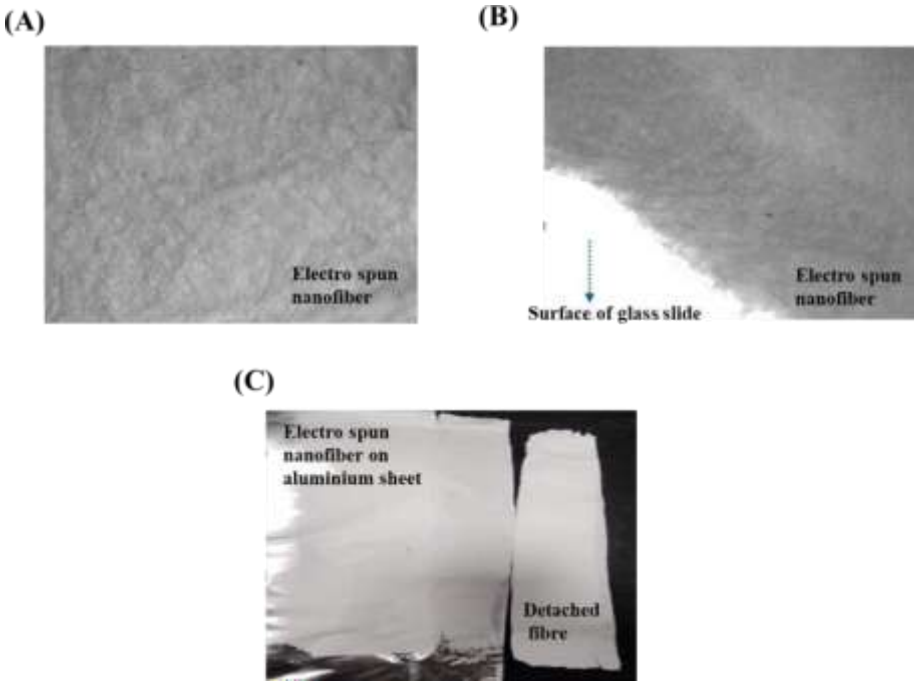
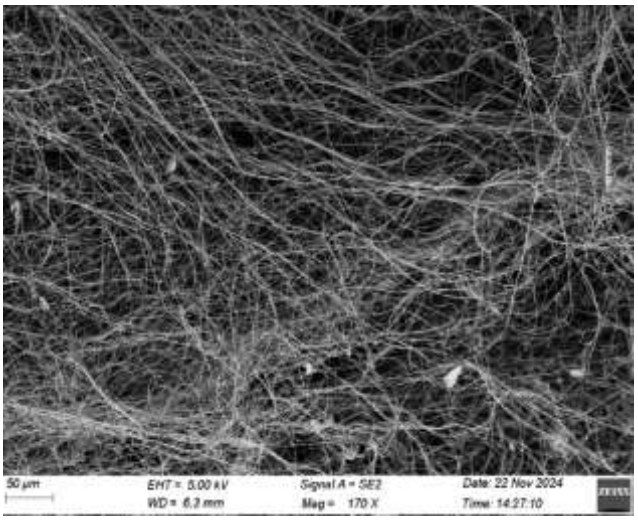


Fig. 5.1.: Preparation of 25 % w/v Type-A Gelatin Electrospun Nanofiber **(A)** 20X image of electrospun nanofiber under inverted microscope, **(B)** Image showing distinction between electrospun nanofiber and glass slide surface showing edge of fibre under inverted microscope, **(C)** Image showing electrospun nanofiber on aluminium sheet and detached nanofiber from aluminium sheet

5.2.2. SEM characterization of electrospun nanofibre

SEM imaging was done by gold-coating of gelatin nanofiber and visualization under the instrument. The 50 µm scale of nanofiber shows even distribution of nanofibers with only a few clumps formation indicating good fibre formation as in **Fig. 5.2. (A)**, and **Fig. 5.2. (B)**.

(**A**)



(**B**)

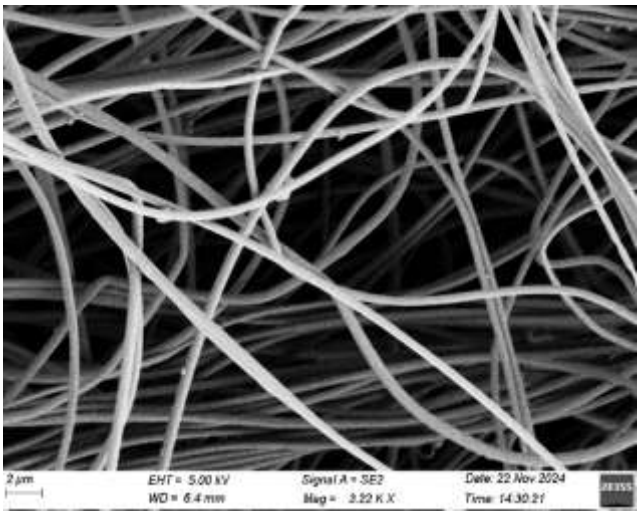


Fig. 5.2.: SEM image of Type-A Gelatin nanofibres. **(A)** Image of nanofiber at 50 µm scale bar before cross linking. **(B)** Image of nanofiber at 2 µm scale bar before cross-linking. The average diameter of the nanofiber is **347 ± 51 nm**.

5.2.3. Cross-linking of gelatin electrospun nanofiber

Gelatin nanofiber was vapor treated with 25% glutaraldehyde for 12 hours. The SEM results of cross- linked nanofiber show increased diameter of nanofiber to around 900 ± 107 nm probably due to moisture uptake from glutaraldehyde vapour. Enzyme activity was evaluated for cross-linked nanofiber and then washed with distilled water. **(Fig. 5.3.)**

After the first wash, excess enzyme washed off and only cross-linked enzyme remained bound to the nanofiber. By evaluating the change in enzyme activity, it was evaluated that crosslinking efficiency of enzyme on nanofiber was found to be **54 ± 2.5%**

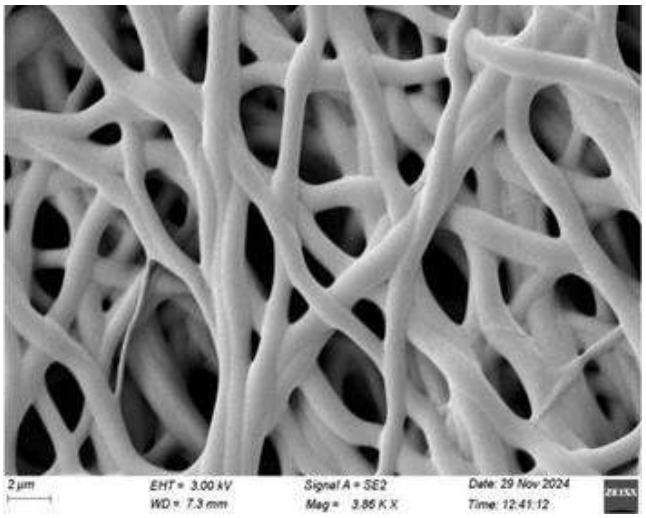


Fig. 5.3.: SEM image of cross-linked gelatin nanofiber using glutaraldehyde vapor treatment.

5.2.4. Detection of Ethyl Paraoxon using Nus-OPH loaded Gelatin nanofibre by Microplate reader

During the reaction, a yellow color is observed, signifying the formation of para-nitrophenol (PNP), a product of the enzymatic degradation of the organophosphate compound, Ethyl Paraoxon. The intensity of the yellow color in the solution correlates with the concentration of PNP generated, thereby indicating the level of Ethyl Paraoxon (EP) present in the reaction mixture. The progressive increase in color intensity serves as a clear visual marker of enzymatic activity and the efficient degradation of the organophosphate pesticide into its byproducts. A linear relationship between absorbance and EP concentration was established, with an R^2 value of 0.9925, confirming the reliability of the measurements. The EP concentrations ranged from 0 µM to 500 µM, and absorbance values were recorded using a SYNERGY H1 microplate reader at 410 nm. These results

support the effectiveness of the enzymatic process in breaking down the pesticide. **(Fig. 5.4.)**

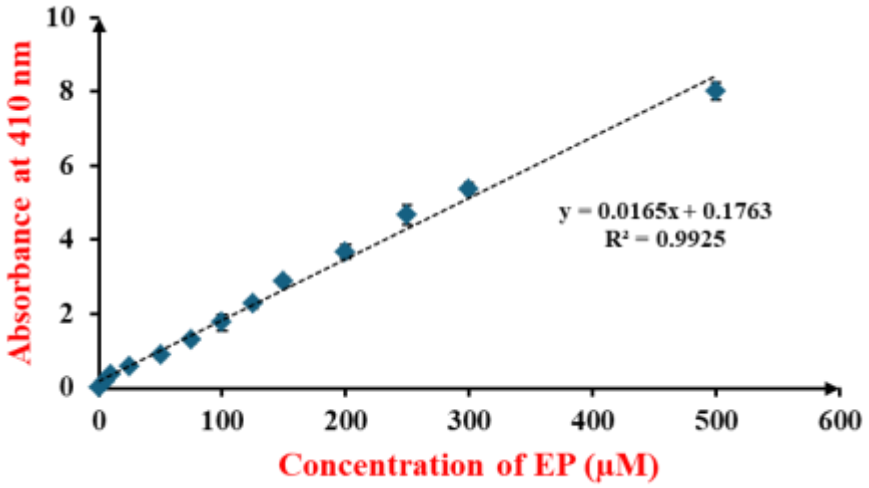


Fig. 5.4.: Degradation of EP by Nus-OPH enzyme. A linear relationship was established between the EP concentration and the fluorescence intensity of PNP, with an R^2 value of 0.9925

5.2.5. Detection of Coumophos using Nus-OPH loaded Gelatin Nanofibre by Microplate reader

A reaction was carried out to study the degradation of Coumaphos (CM) into its byproducts, diethylthiophosphoric acid (DETP) and chlorferon. Among the byproducts, chlorferon exhibits fluorescent properties, with an absorption peak at 370 nm and an emission peak at 470 nm using SYNERGY H1 microplate reader. The fluorescence of chlorferon was monitored as an indicator of the enzymatic breakdown of Coumaphos, with higher fluorescence intensity reflecting greater degradation into its byproducts. A linear relationship was established between fluorescence intensity and Coumaphos concentration, demonstrating an R^2 value of 0.9863, which indicates the reliability of the measurements. The concentrations of Coumaphos varied from 0 µM to 150 µM. These findings highlight the

efficiency of the enzymatic process in degrading the pesticide and provide quantitative evidence of its effectiveness. **(Fig. 5.5.)**

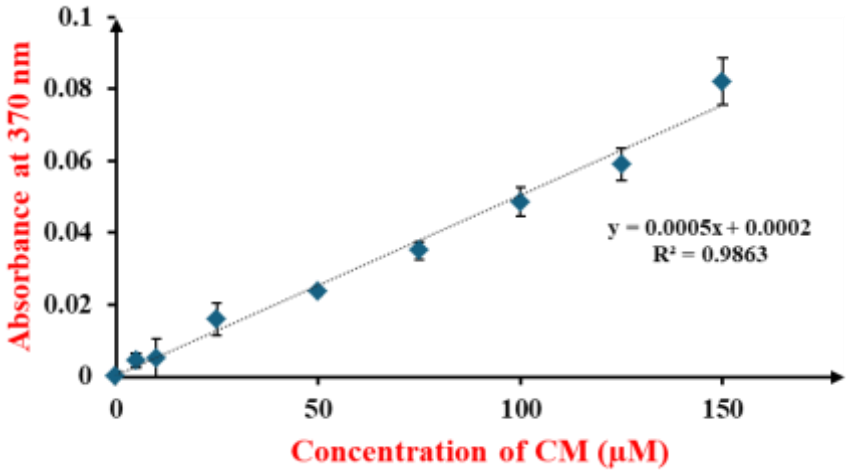


Fig. 5.5.: Degradation of Coumaphos by Nus-OPH and formation of Fluorescent Byproduct Chlorferon, with a linearity of 0.9863

Table 5.1: Systematic Table showing various parameters when there is degradation of OP compound.

Substrate	Linear range	Linearity	LOD	LOQ	Resolution	Sensitivity
Ethyl paraoxon	5-500 µM	0.9925	2.5 µM	7.5 µM	0.75 µM	0.0165 a.u./ µM
Coumaphos	5 – 150 µM	0.9863	13.2 µM	39.6 µM	4 µM	0.0005 a.u./ µM

5.2.6. Calibration curve of EP using HPLC

High-Performance Liquid Chromatography (HPLC) is utilized to create a standard calibration curve for Ethyl Paraoxon (EP). This calibration curve served as a crucial reference for our analysis. Using this method, the aim was to monitor the depletion of EP as the substrate in the reaction and to track the formation of para-nitrophenol (PNP) as a product of EP degradation as shown in **Fig. 5.6.**

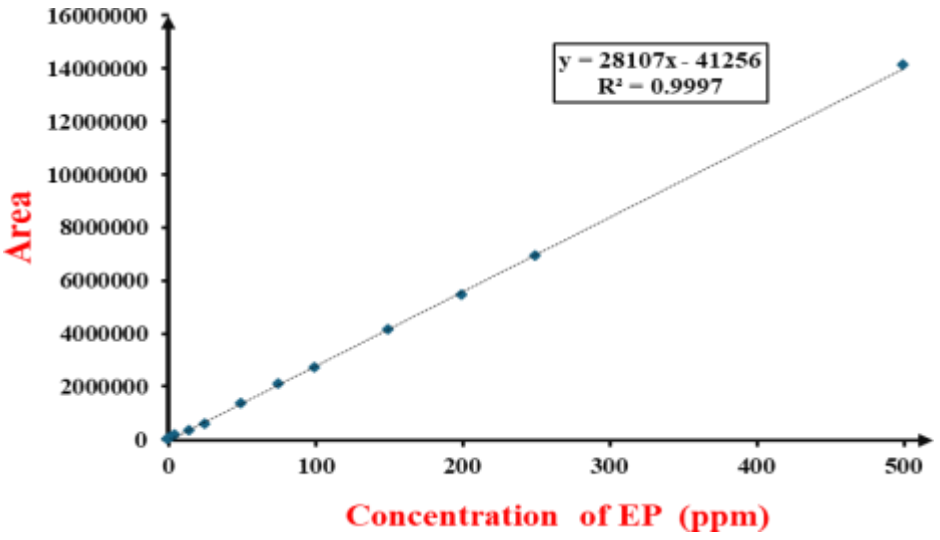


Fig. 5.6.: Calibration curve of EP obtained via HPLC and 0.99 as the R^2 value.

The standard calibration curve allowed us to quantify the exact concentrations of EP and PNP during the experiment. By comparing the observed peaks and their intensities on the chromatograms with the calibration data, determination of how much EP was consumed and how much PNP was formed was done as shown in **Fig. 5.7.**

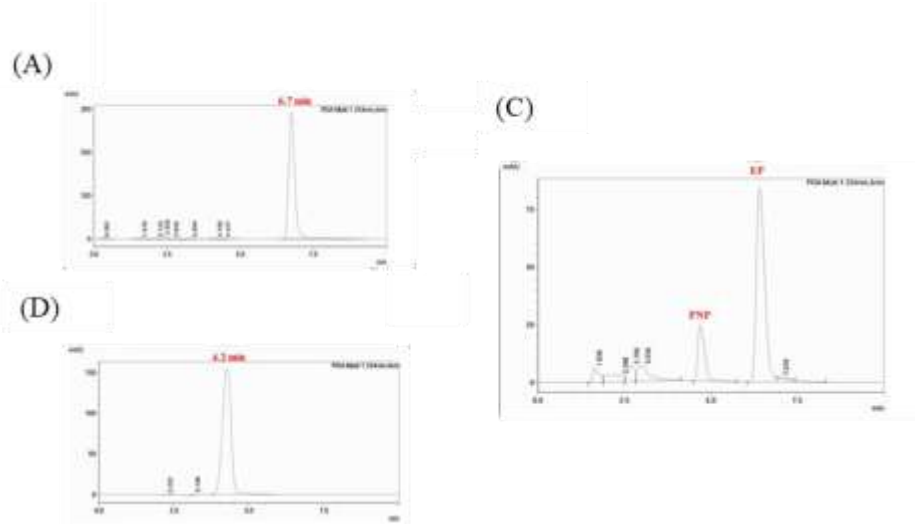


Fig. 5.7.: HPLC Chromatograms of **(A)** EP (standard) **(B)** PNP (standard) **(C)** EP degradation into PNP by Nus-OPH loaded Gelatin Nanofibres after 15 minutes.

5.2.7. Bioremediation of Ethyl Paraoxon using Nus-OPH loaded Gelatin nanofibers

An experiment was conducted using both the free enzyme and the enzyme-loaded gelatin nanofiber. First, a reaction mixture was prepared and added the free enzyme as well as the enzyme- loaded nanofibers separately. After incubating the mixture for 10 minutes, the results were using HPLC and observed distinct peaks. Our findings showed that the free enzyme degraded 99% of Ethyl Paraoxon (EP) within just 30 minutes. In comparison, the Nus-OPH loaded gelatin nanofibers achieved 85% degradation of EP within 1 hour. This difference can be explained by the fact that some of the enzyme became embedded within the nanofibers, which limited its availability on the surface to degrade EP effectively as demonstrated by **Fig. 5.8.**

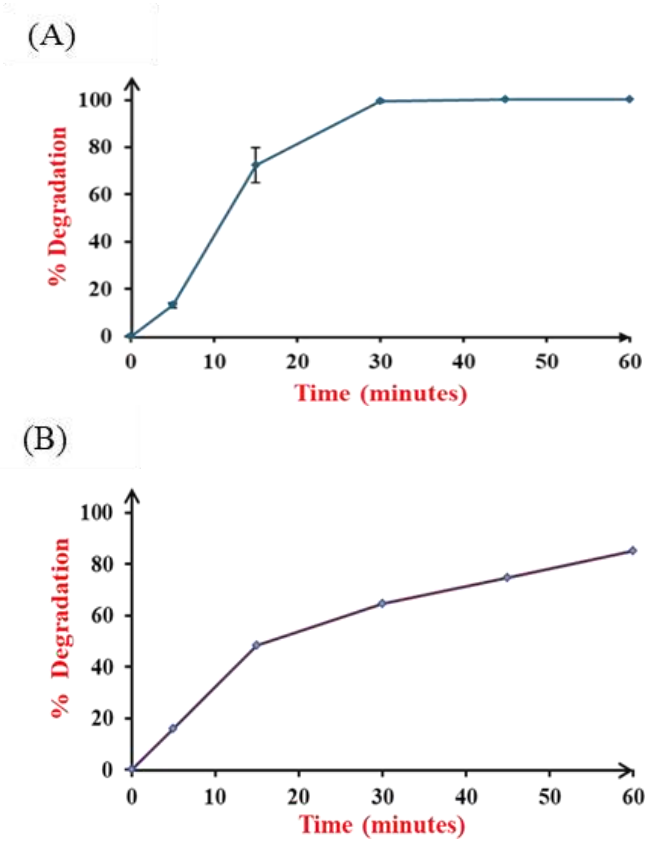


Fig. 5.8.: Bioremediation by using **(A)** free enzyme showing 99 % of EP degradation within 30 minutes. **(B)** Nus-OPH loaded gelatin nanofibre which shows 85 % of EP degradation in 1 hour. The enzyme unit used was 0.03 U.

5.2.8. Calibration curve of Chloreferon using HPLC

High-Performance Liquid Chromatography (HPLC) is utilized to create a standard calibration curve for Chloreferon (CM), which is the byproduct of Coumaphos degradation. This calibration curve served as a crucial reference for our analysis. Using this method, the aim was to monitor the depletion of CM as the substrate in the reaction and to track the formation of Chloreferon as a product of CM degradation as shown in **Fig. 5.9.**

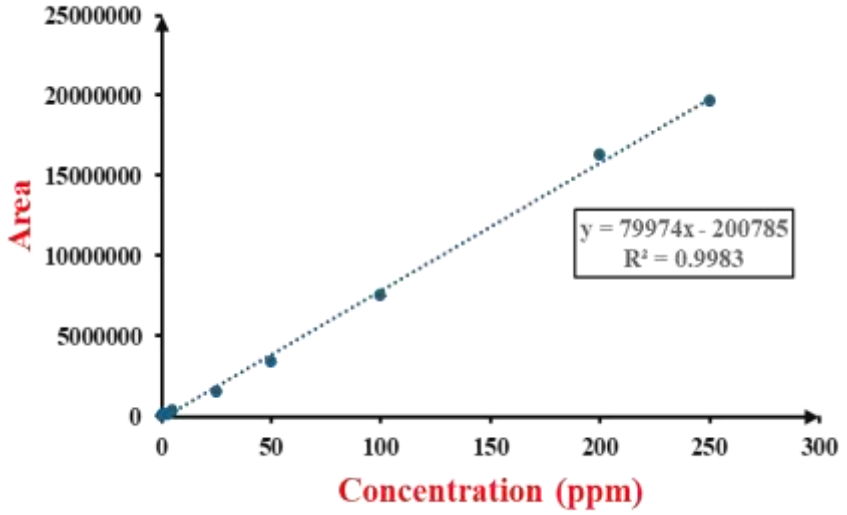


Fig. 5.9.: Calibration curve of Chloreferon obtained via HPLC and 0.99 as the R^2 value.

The standard calibration curve allowed us to quantify the exact concentrations of Chloreferon during the experiment. By comparing the observed peaks and their intensities on the chromatograms with the calibration data, determination of how much CM was consumed and how much Chloreferon was formed was done as shown in **Fig. 5.10.**

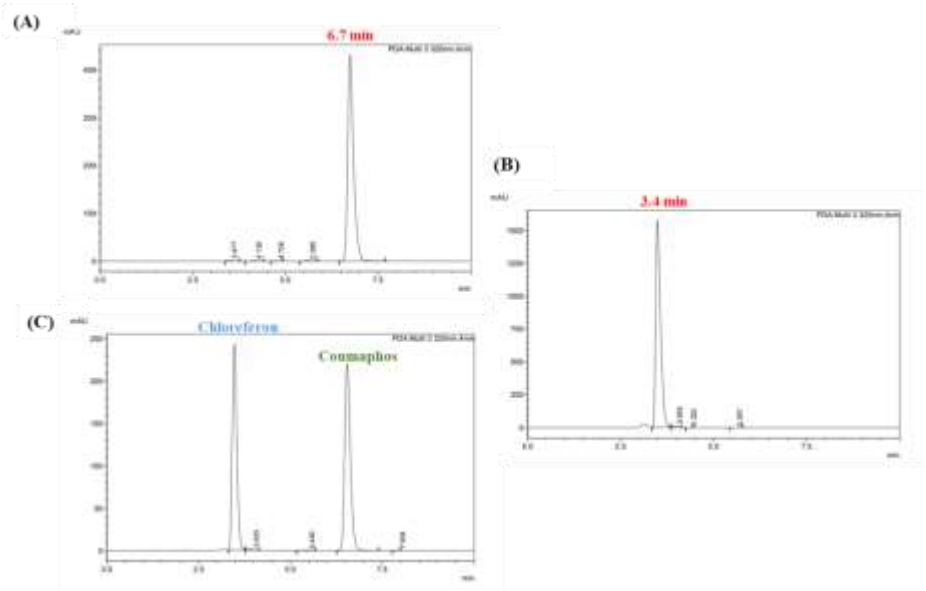


Fig. 5.10.: HPLC Chromatograms of (A) CM (standard) (B) HPLC chromatogram of Chloreferon (C) CM degradation into Chloreferon by Nus-OPH loaded Gelatin nanofibers after 30 minutes.

5.2.9. Bioremediation of CM using Nus-OPH loaded Gelatin nanofibers

An experiment was conducted using the enzyme-loaded gelatin nanofibers. First, a reaction mixture (50 mM CM, 0.015 U of Nus-OPH) was prepared and added the enzyme- loaded nanofibers separately. After incubating the mixture for 10 minutes, the results were using HPLC and observed distinct peaks. Our findings showed that the Nus-OPH loaded gelatin nanofiber achieved 99% degradation of CM in 2 hours as demonstrated by **Fig. 5.11.**

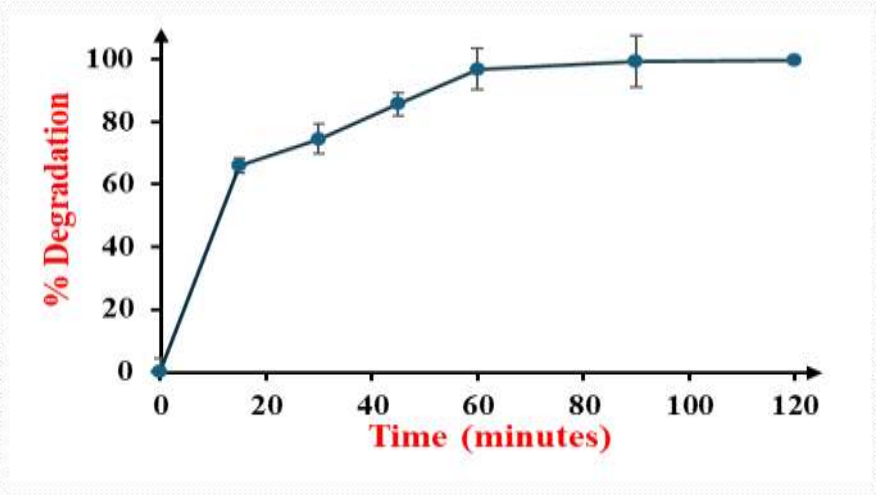


Fig. 5.11.: Bioremediation by using Nus-OPH loaded gelatin nanofibre which shows 99 % of CM degradation in 2 hours

5.2.10. Reusability of enzyme linked nanofibers checked with Ethyl Paraoxon degradation

To check efficient crosslinking of Nus-OPH enzyme on gelatin nanofibers, multiple enzyme assay cycles were conducted with Ethyl Paraoxon. Washes with 10 mL of distilled water was provided in between subsequent cycles. The result demonstrates that around 90 % of enzyme activity is retained after fifth wash highlighting efficient cross-linking of enzyme on gelatin nanofiber as shown in **Fig. 5.12.**

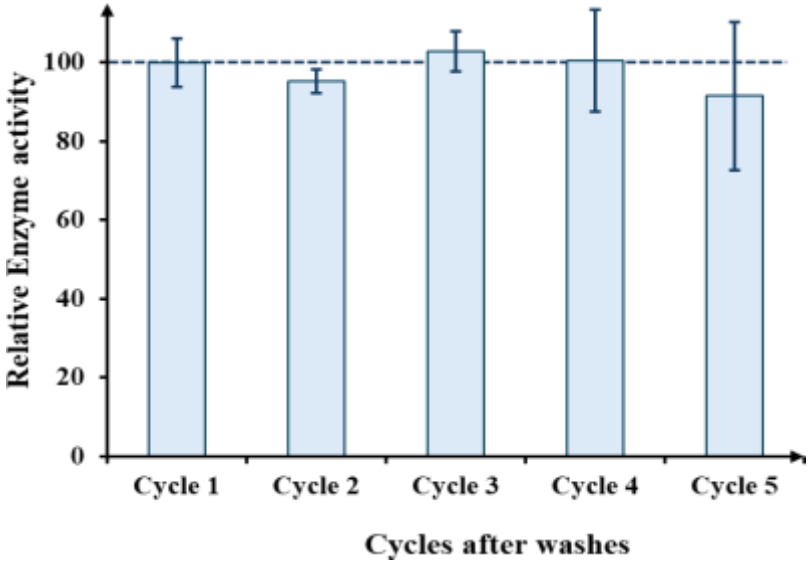


Fig. 5.12.: Nus-OPH enzyme cross-linked with Nano-fibre showing almost 90 % of enzyme stability after five washes.

5.2.11. Reusability of enzyme linked nanofibers checked by Caumophos degradation

To check efficient crosslinking of Nus-OPH enzyme on gelatin nanofibers, multiple enzyme assay cycles were conducted with Caumophos degradation. Washes with 10 mL of distilled water was provided in between subsequent cycles. The result demonstrates that around 90 % of enzyme activity is retained after fifth wash (using SYNERGY H1 microplate reader) highlighting efficient cross-linking of enzyme on gelatin nanofiber as shown in **Fig. 5.13.**

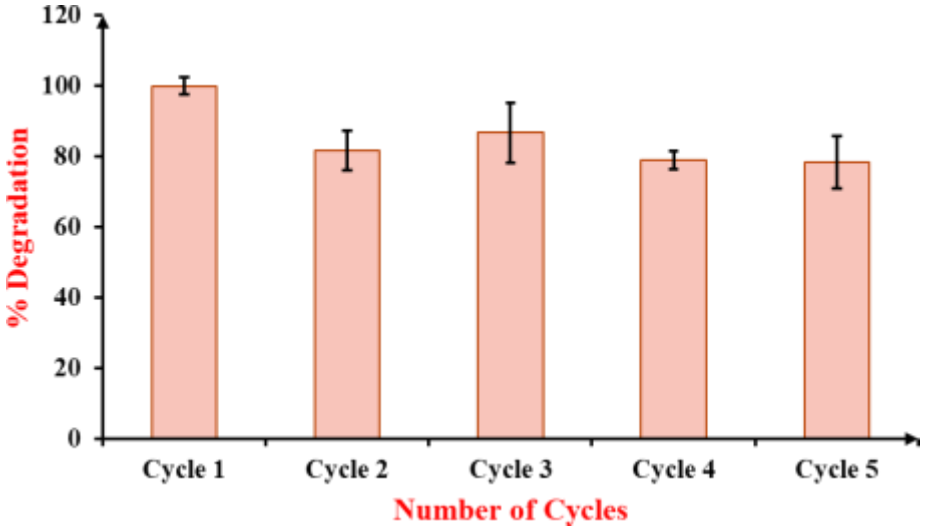


Fig. 5.13.: Nus-OPH enzyme cross-linked with nanofiber showing almost 80 % of enzyme stability after five washes.

5.3. Summary

Electrospun nanofibers have shown great potential for pesticide degradation due to their structural and functional features. These nanofibers have a high surface area-to- volume ratio and a porous design, allowing for efficient pesticide interaction. They are functionalized with active agents such as enzymes such as OPH, which improve degradation processes while preserving stability and reusability. Gelatin-based electrospun nanofibers cross- linked with glutaraldehyde were successfully used to detect and degrade organophosphate pesticides, such as EP and CM. The study proved reusability by observing numerous cycles of pesticide breakdown. This study demonstrates the effectiveness of enzyme-functionalized electrospun nanofibers as eco-friendly pesticide detection and degradation tools, paving the path for advanced environmental remediation and sustainable agriculture practices.

Chapter 6

Enzyme immobilized ready-made textile for Organophosphate Compound Detoxification

6.1. Introduction

Pesticides are crucial in farming because they improve crop quality and output. However, overuse and exposure can cause major health problems. Farmers who spray pesticides in fields are frequently exposed to hazardous organophosphate (OP) compounds. Many cases of OP poisoning have been reported, which is causing alarm among agricultural workers (Aktar et al., 2009). Farmers frequently become accidentally exposed to OP compounds while spraying crops. This can produce acute symptoms such as nausea, vomiting, and respiratory distress. In severe circumstances, it may lead to hospitalization. A case in Europe, an 85-year-old man was accidently exposed to dimethoate, an OP chemical, through multiple routes (inhalation, skin contact, etc.). He had acute respiratory failure but survived with intensive care. In developing countries, OP compounds are intentionally consumed as a form of self-harm (Scaria et al., 2023).

In this study, a method was developed to address the issue of OP contamination on ready-made textiles. An enzyme called Nus-OPH was applied straight to the fabric. This enzyme has the ability to convert hazardous OP chemicals into less poisonous ones. To ensure that the enzyme remains stuck on the fabric, its reusability is evaluated after numerous washing cycles to ensure it was still effective. Aside from breaking down the OP chemicals, we also tried to identify their presence on the fabric. For this, we employed a technique known as RGB analysis, which is easy and quick. It functions as a point-of-care (POC) instrument, making it easy to determine the concentration of OP chemicals sprayed on textiles. This approach not only aids in the degradation of OP compounds, but also allows for their detection,

making it a practical and efficient alternative for pesticide residue management on textiles

6.2. Results and Discussions

6.2.1. Degradation of Organophosphate compounds from enzyme loaded cotton fabrics

In order to degrade OP compounds (Ethyl paraoxon and Coumaphos), Nus-OPH of 15µg was loaded on cotton fabrics while for degradation of Ethyl paraoxon by OPAA- FL, an amount of 1000 µg of protein was loaded on cotton fabrics. (**Fig. 6.1.**)

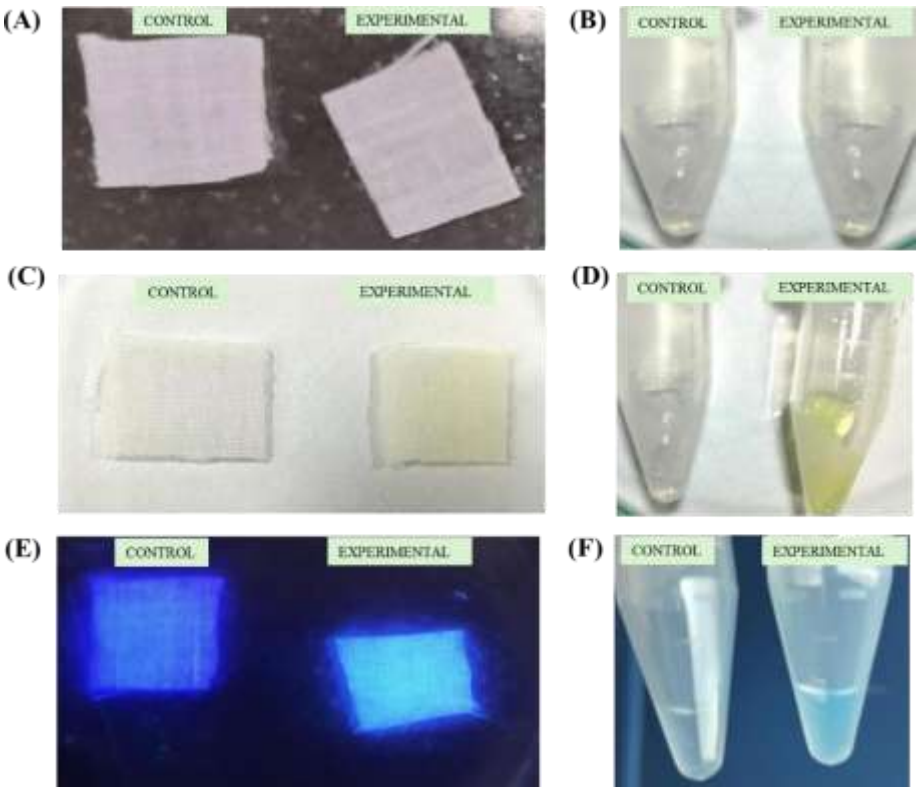


Fig. 6.1.: Enzyme loaded cotton fabrics for efficient degradation of 100 ppm of OP compounds. **(A)** Clothes before incubation in OP compounds **(B)** Enzyme reaction at 0 minutes **(C)** Clothes after incubation in 100 ppm of EP after 180 minutes **(D)** Enzyme reaction after 180 minutes **(E)** Clothes after incubation in 100 ppm of Coumaphos after 180 minutes **(F)** Enzyme reaction after 180 minutes.

6.2.1. RGB analysis (Directly on readymade textile)

Small pieces of cloth were prepared, each carefully cut into dimensions of 1×1 cm. These cloth pieces served as a platform for our OP-degrading enzyme, Nus-OPH, which we applied and then allowed to air-dry at room temperature. Once the enzyme had dried, a reaction mixture was prepaperd containing various concentrations of Ethyl Paraoxon (EP), as illustrated in the accompanying **Fig. 6.2.**

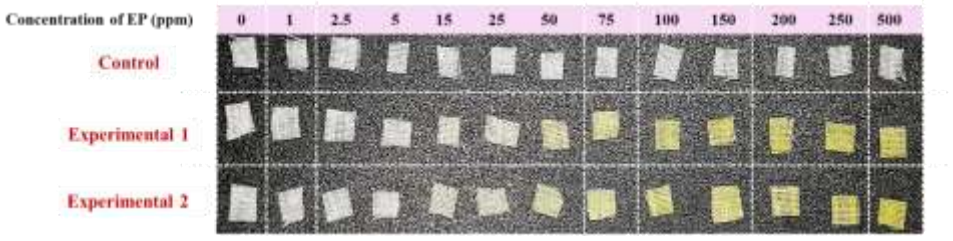


Fig. 6.2.: Detection of EP on cloth with varying concentrations of EP using RGB analysis.

The reaction mixture was evenly applied to the enzyme- immobilized cloth pieces, ensuring proper distribution, and the samples were incubated at 37°C. To analyze the results, the "Color Grab" software was used to capture the values of the three primary colors: Red, Green, and Blue (RGB). Different combinations of these values were explored to identify the most consistent trends. Through this approach, we determined that the blue color (**Fig. 6.3.**) and the Red/Blue combination (**Fig. 6.4.)** provided the most reliable indicators. In the context of RGB analysis, the primary colors— Red, Green, and Blue— play a crucial role. During the degradation of EP, we observed the formation of para- nitrophenol (PNP), which exhibits a yellow color. The intensity of this yellow color varied depending on the concentration of EP. The presence of yellow signifies that the blue component of light has been absorbed, resulting in distinct values for red and green, with no contribution from blue. However, in lighter shades of yellow, there can still be some blue values observed.

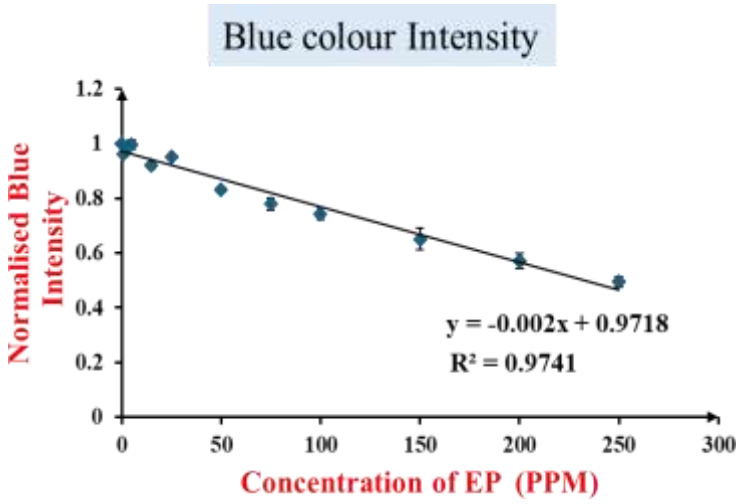


Fig. 6.3.: Trend based on Blue Color Intensity as shown by RGB analysis using a software called Color Grab.

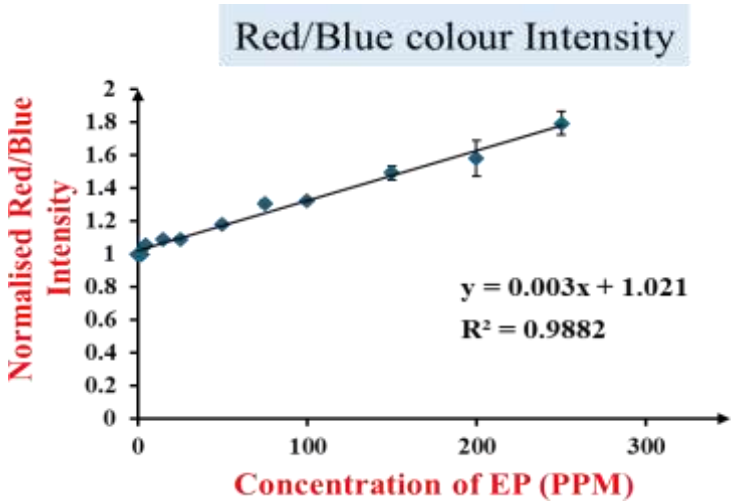


Fig. 6.4.: Trend based on Red/Blue Color Intensity as shown by RGB analysis using a software called Color Grab.

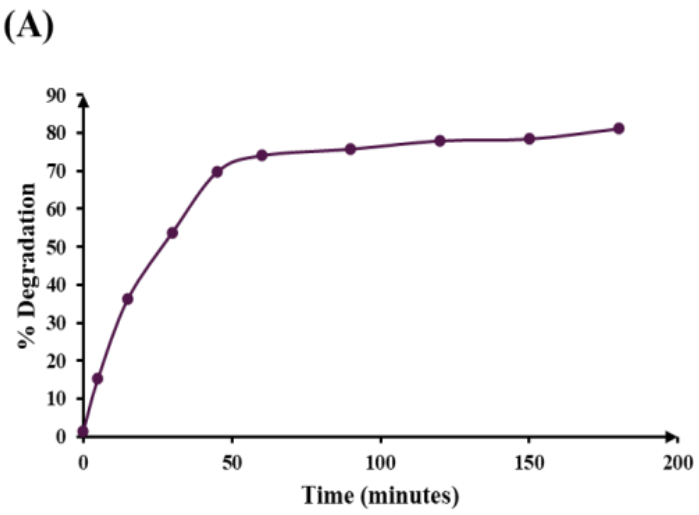
A comparative analysis of RGB values involving different parameters of detection as summarized in the **Table 6.1.**

Table 6.1.: RGB analysis showing various parameters of detection of EP

RGB	LOD	LOQ	Sensitivity	Resolution	% RSD	Linear Range
BLUE Color Intensity	3.68 ppm	11.15 ppm	0.002 intensity/ ppm	2.5 ppm	3.30 %	0 to 250 ppm
RED/BLUE Color Intensity	7.04 ppm	21.12 ppm	0.003 intensity/ ppm	2.13 ppm	4.60 %	0 to 250 ppm

6.2.2. Ethyl Paraoxon degradation on cotton textile fabric by Nus-OPH

In tap water, Nus-OPH enzyme loaded in a fabric, showed about 80 % degradation of 100 ppm of Ethyl Paraoxon within 180 minutes. While in suitable buffering condition (CHES buffer, pH 9.0), Nus-OPH loaded fabric showed 100% degradation of 100 ppm of Ethyl Paraoxon within 60 minutes (**Fig. 6.5.**).



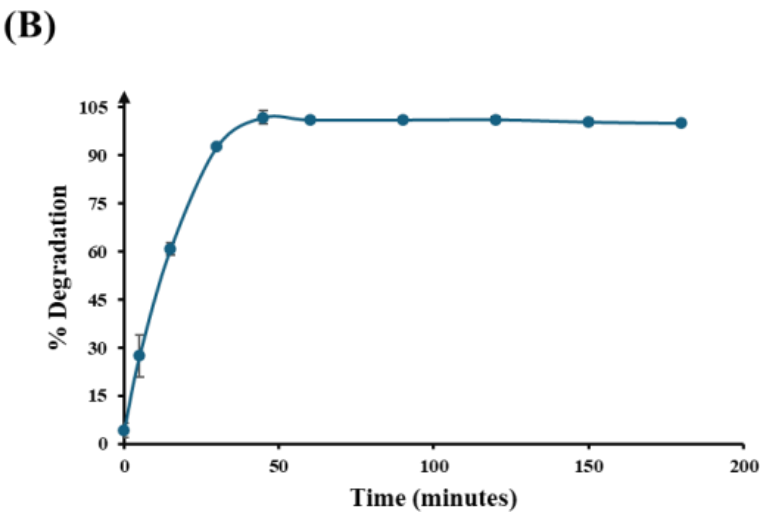


Fig. 6.5.: Degradation of 100 ppm of Ethyl Paraoxon using Nus- OPH loaded cotton fabrics. **(A)** In Tap Water, **(B)** In CHES Buffer (pH-9.0)

6.2.3. Coumaphos degradation on cotton textile fabric by Nus-OPH

In tap water, Nus-OPH enzyme loaded in a fabric, showed about 48 % degradation of 100 ppm of Coumaphos within 180 minutes. While in suitable buffering condition [CHES buffer- pH 9.0], Nus-OPH loaded fabric showed 45% degradation of 100 ppm of Ethyl Paraoxon within 180 minutes. Complete hydrolysis of Coumaphos did not take place due to less concentration of Nus-OPH enzyme as it has lower affinity for Coumaphos in comparison to ethyl paraoxon. (**Fig. 6.6.**)

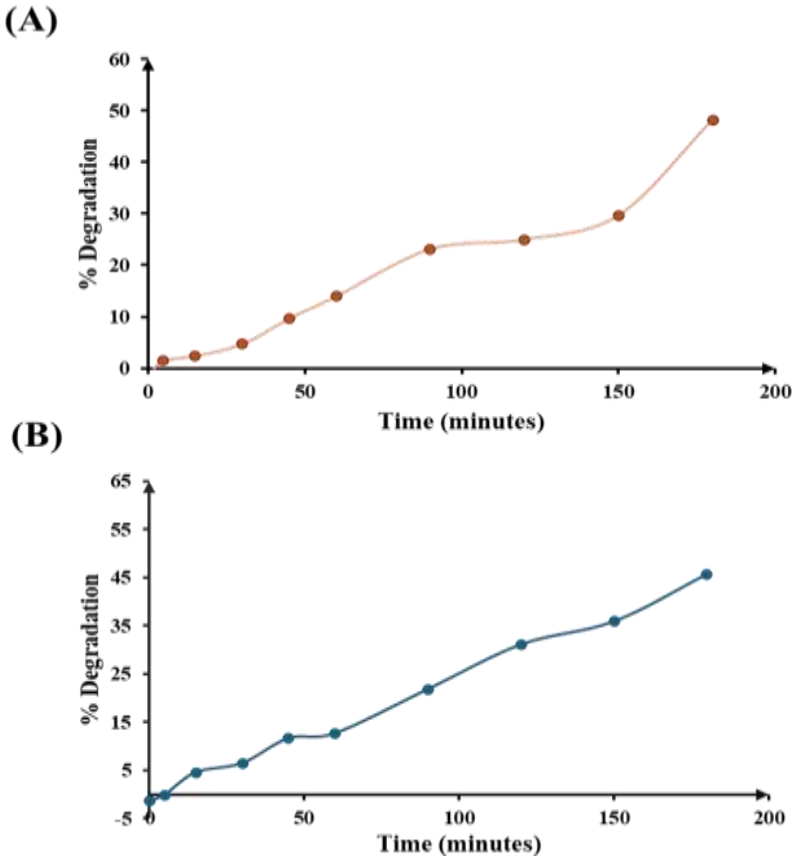


Fig. 6.6.: Degradation of 100 ppm of Coumaphos using Nus- OPH loaded cotton fabrics. **(A)** In Tap Water, **(B)** In CHES Buffer (pH-9.0)

6.2.4. Ethyl Paraoxon degradation on textile by OPAA-FL

In tap water, OPAA-FL enzyme loaded in a fabric, showed about 80% degradation of 100 ppm of Ethyl Paraoxon within 180 minutes. While in suitable buffering condition (BTP buffer, pH 8.5), OPAA-FL loaded fabric showed 95% degradation of 100 ppm of Ethyl Paraoxon within 180 minutes as depicted in **Fig. 6.7.**

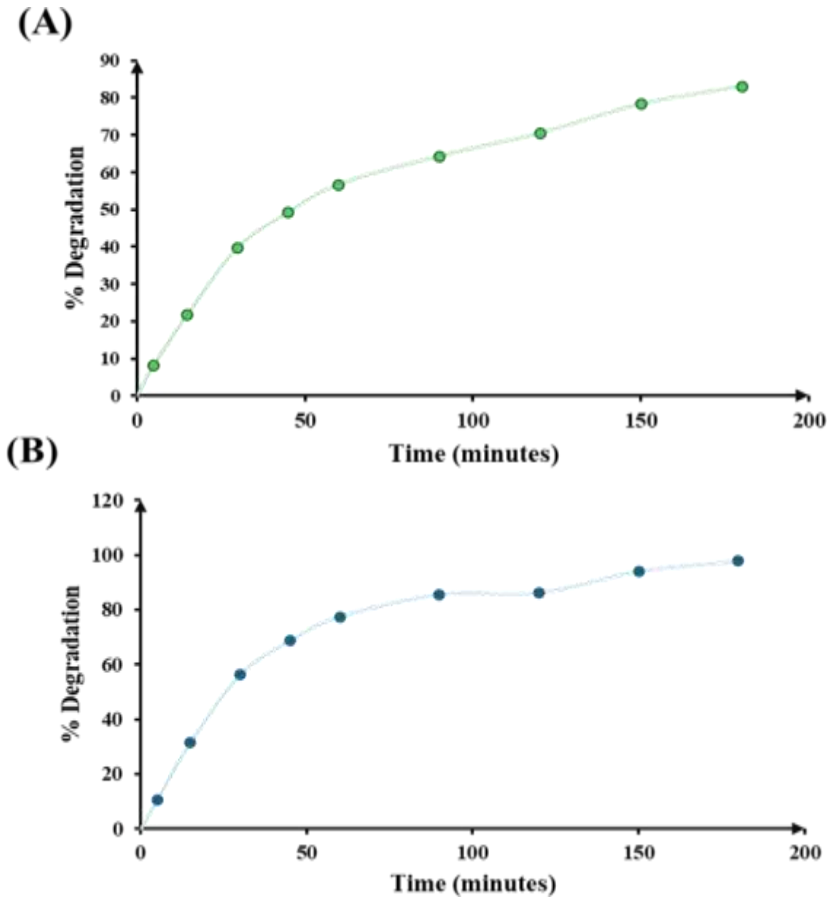


Fig. 6.7.: Degradation of 100 ppm of Ethyl Paraoxon using OPAA-FL loaded cotton fabrics. **(A)** In Tap Water, **(B)** In BTP Buffer (pH-8.5)

6.2.5. Reusability of readymade cotton textile

To check efficient crosslinking of Nus-OPH enzyme on readymade cotton textile, multiple enzyme assay cycles were conducted with Ethyl Paraoxon. Washes with 10 mL of distilled water was provided in between subsequent cycles. The result demonstrates that around 85 % of enzyme activity is retained after fifth wash highlighting efficient cross-linking of enzyme on readymade textile as shown in **Fig. 6.8.**

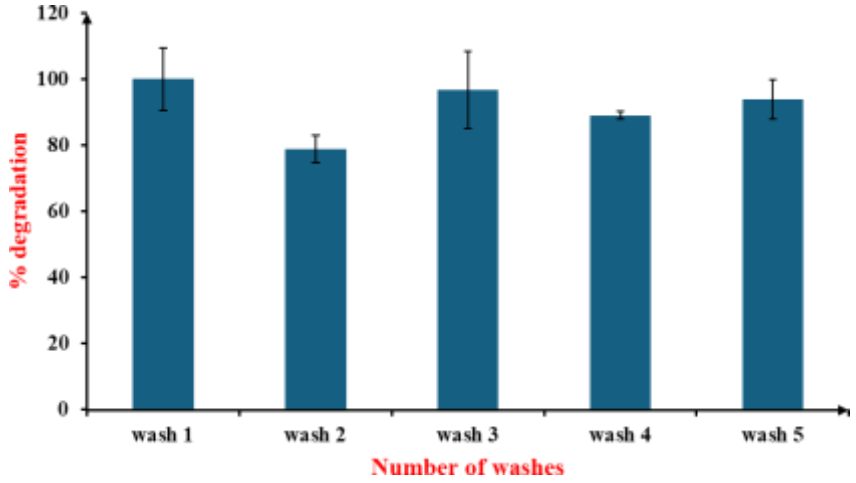


Fig. 6.8.: Nus-OPH enzyme immobilized with cotton textile showing almost 85 % of enzyme stability after five washes.

6.2.6. Detection of Ethyl Paraoxon with varied concentrations using Nus-OPH loaded cotton cloth (Solution phase)

The experiment was begun by taking readymade cotton textile cloth and cutting it into small, uniform pieces measuring 1×1cm. OP-degrading enzyme, Nus-OPH, which was applied on the cloth and left to air-dry at room temperature. To evaluate the enzyme's activity, a reaction mixture was prepared containing various concentrations of Ethyl Paraoxon (EP) as shown **Fig. 6.9.** After applying the reaction mixture to the enzyme-coated cloth pieces, we incubated them for 10 minutes to allow the reaction to proceed. Following the incubation, the cloth pieces were removed, and we measured the absorbance of para-nitrophenol (PNP), a product formed during the degradation of EP, using a plate reader.

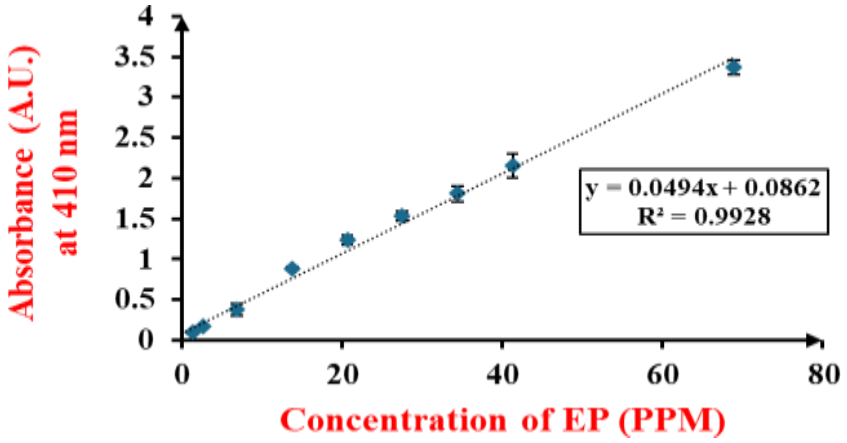


Fig. 6.9.: Detection of Organophosphate compound in solution phase having 0.99 as a R^2 value.

To observe the formation of PNP, we assessed several analytical parameters.

Linearity: Linearity is a function of values that can be represented graphically as a straight line. It can be defined as the capability to show "Results that are directly proportional to the concentration of the analyte in the sample." (Regression Line)

Limit of Detection or LOD: It is described as the smallest amount of analyte in the sample that can be detected, albeit it is not always measured as a precise value.

$$LOD = 3.3 \times \frac{standard\ deviation\ of\ lowest\ point}{slope}$$

Limit of Quantification or LOQ: It is described as the smallest amount of analyte in the sample that can be accurately quantified and measured quantitatively.

$$\mathbf{LOQ = 3*LOD}$$

Sensitivity: It is the slope of the curve and is defined as the minimum detectable response which is generated by the change in concentration.

Resolution: It is the minimum concentration difference that can be detected by the analytical method.

$$Resolution = \frac{standard\ deviation\ of\ lowest\ point}{slope}$$

6.3. Summary

Organophosphate (OP) compounds, which are commonly employed as pesticides in agriculture, pose severe health concerns, particularly to farmers who are repeatedly exposed during pesticide application. Such exposure can result in organophosphate poisoning, which can cause severe symptoms such as nausea and breathing difficulties, as well as nervous system damage or even death. OP residues on textiles heighten the danger of exposure, emphasizing the need for appropriate strategies to control these hazardous compounds. This study aims to address OP contamination in ready-made textiles by immobilizing the enzyme Nus-OPH onto cotton fabric. Nus- OPH is an enzyme that degrades hazardous OP chemicals into less toxic ones. Cotton, which is mostly made of cellulose, has hydroxyl groups are chemically bonded to the enzyme's amine (-NH2) groups, resulting in a stable and long-lasting attachment. This ensures that the enzyme remains active even after several washing cycles. Furthermore, the study used RGB analysis as a detection tool to determine the concentration of OP compounds on textiles. This simple and user-friendly technique is a useful tool for managing pesticide contamination. Together, these technologies provide a novel, dual approach strategy to increasing textile safety and lowering pesticide exposure concerns.

Chapter 7

Enzyme Loaded Alginate Nanoparticle for Organophosphate Compound Detoxification

7.1. Introduction

Organophosphate poisoning is a major public health hazard, with many instances recorded worldwide as a result of industrial exposure or unintentional consumption. Symptoms include nausea, breathing difficulties, and severe neurological abnormalities. This study identifies a promising technique for reducing OP toxicity, paving the path for safer, more environmentally friendly interventions to combat pesticide-related poisoning in humans (Reddy et al., 2020). We used sodium alginate nanoparticles encapsulating the Nus-OPH enzyme to degrade organophosphate (OP) chemicals. Using the HEK 293 cell line, researchers assessed the toxicity of Ethyl Paraoxon (EP). Both the free enzyme and the enzyme enclosed in sodium alginate nanoparticles were examined, and the encapsulated enzyme demonstrated higher degrading efficiency. Cellular absorption of sodium alginate nanoparticles was discovered, demonstrating their potential to destroy OP compounds within cells. This novel technique shows promise for detoxifying OP chemicals in humans after unintentional inhalation, providing a viable solution to organophosphate poisoning.

7.2. Results and discussions

7.2.1. Preparation of enzyme loaded sodium alginate nanoparticle

Sodium lginate nanoparticle is made successfully encapsulating enzyme called OPH. After performing enzyme assay there is the formation of yellow colour after 10 minutes of incubation at 37 °C which shows the formation of PNP according to the reaction shown in **Fig. 7.1.**

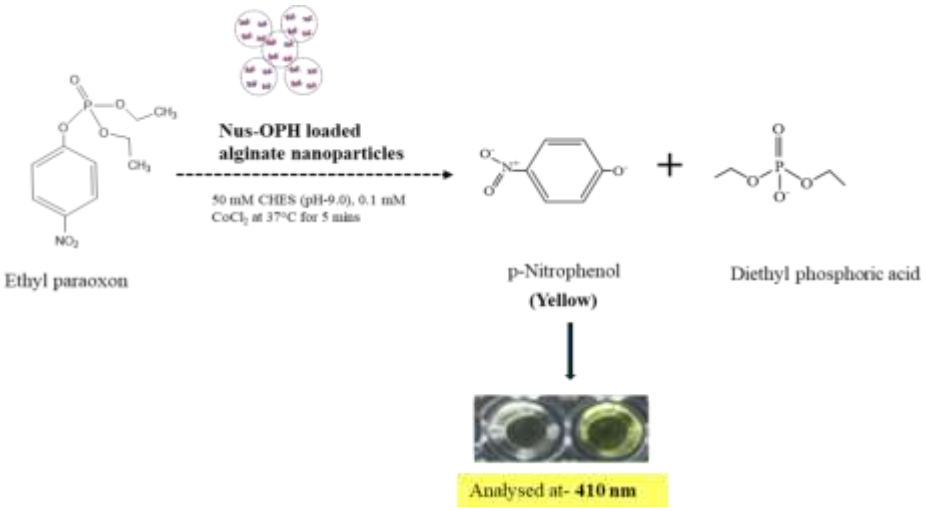


Fig. 7.1.: Chemical reaction showing Ethyl Paraoxon degradation and production of yellow coloured *p*- Nitrophenol (pNP) using enzyme loaded alginate nanoparticles.

7.2.2. Characterisation of Nus-OPH loaded sodium alginate nanoparticles

We obtained a scanning electron microscopy image of Nus-OPH loaded nanoparticles, with EHT of 5.0 KV and a scale range of 200 nm. The nanoparticles were observed in sizes ranging in nano scale (**Fig. 7.2.**).

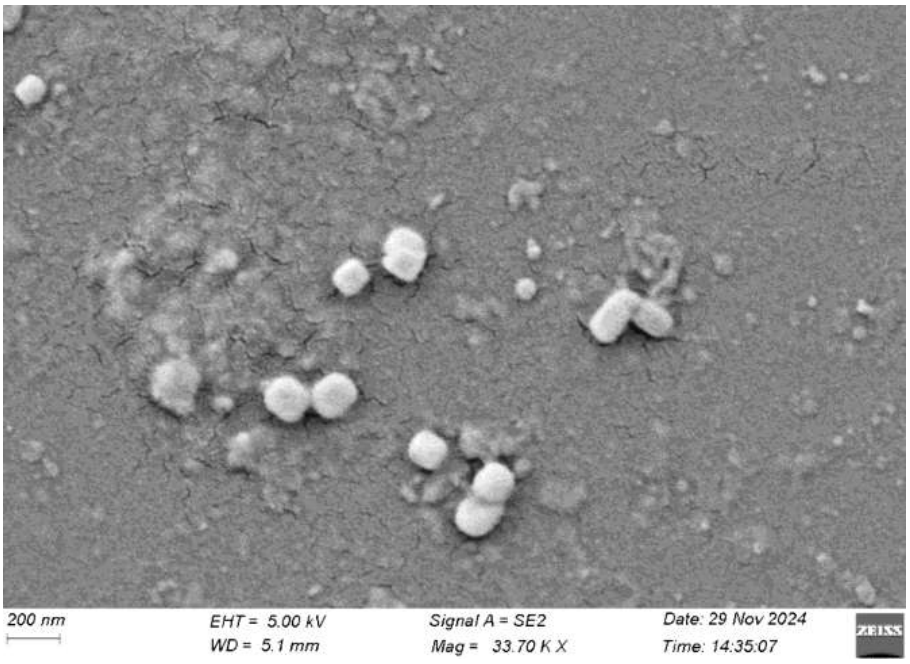


Fig. 7.2.: SEM image of sodium alginate nanoparticles encapsulated Nus-OPH enzyme.

The size distribution curve of the sodium alginate nanoparticles encapsulating Nus-OPH enzyme showed the average size of nanoparticles comes around to be **120.9 ± 49 nm** as shown in **Fig. 7.3.**

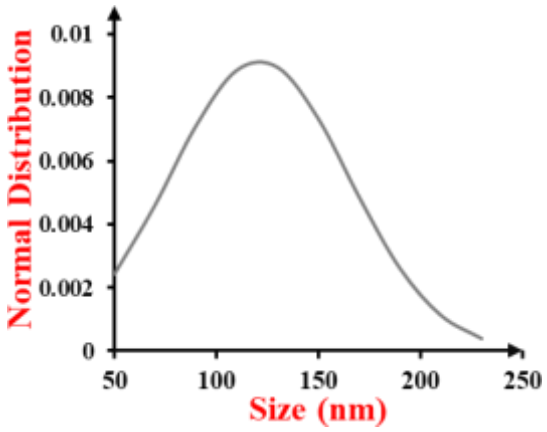


Fig. 7.3.: Size Distribution Curve obtained using SEM and analyzed using ImageJ software.

Then, to confirm the nanoparticles, Dynamic Light Scattering (DLS) was done. This technique is used to determine the size distribution profile of small particles in suspension or polymers in solution. The average size of nanoparticles comes around **210 ± 39 nm** as shown in **Fig. 7.4.**

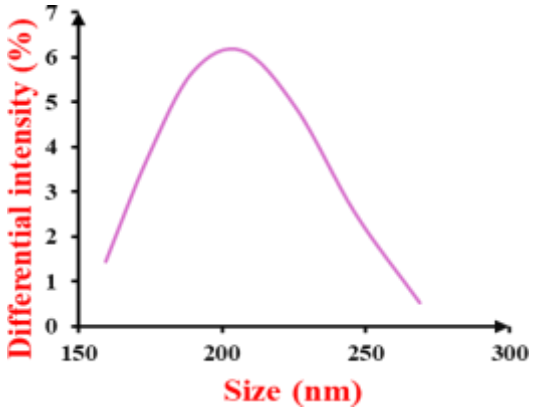


Fig. 7.4.: Dynamic Light Scattering (DLS) of Nus-OPH loaded Sodium Alginate Nanoparticles.

To evaluate enzyme efficiency of Nus-OPH loaded nanoparticles, the enzyme activity for Ethyl paraoxon for free enzyme was 2.35 U/mL while encapsulated nanoparticle showed an enzyme activity of 0.16 U/mL **(Fig. 7.5.)**. Similarly, enzyme activity of coumaphos for

free enzyme was around 0.03 U/mL while encapsulated enzyme showed enzyme activity of 0.0053 U/mL. These results suggest that enzyme loading efficiency falls into range of 5.6 ± 0.5%.

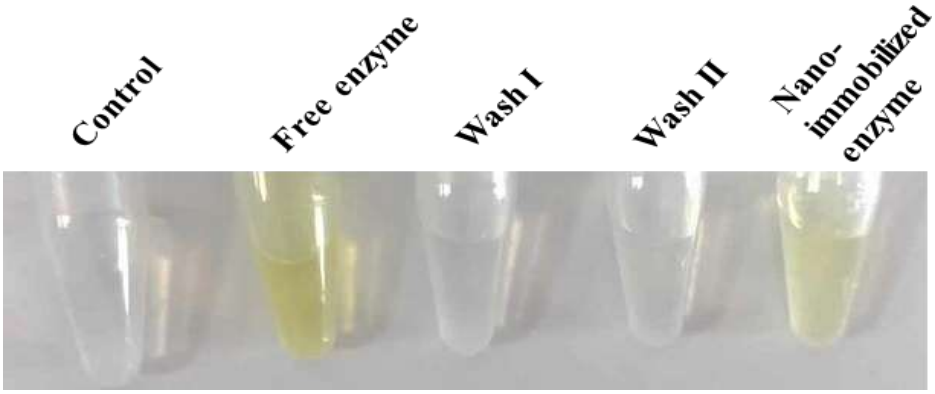
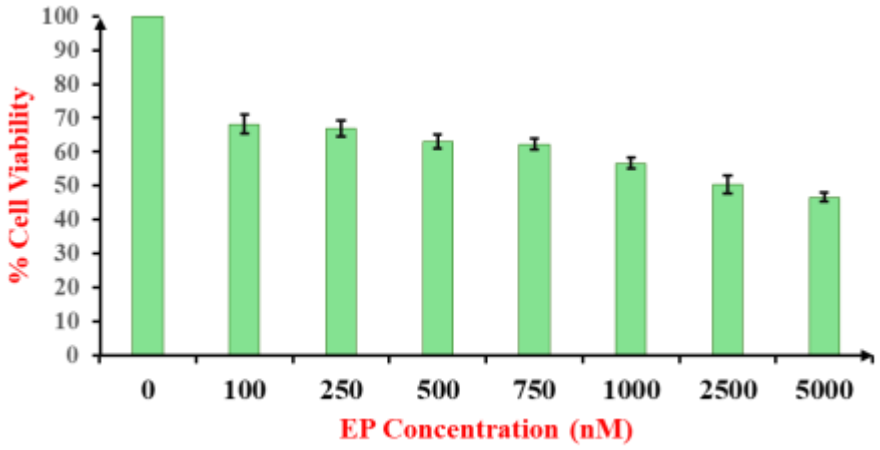


Fig. 7.5.: Degradation of nano-immobilized Nus-OPH alginate nanoparticles for efficient degradation of Ethyl paraoxon.

7.2.3. MTT Cell Viability Assay

The cell viability of HEK-293 cells was assessed when exposed to Ethyl Paraoxon (EP) with IC_{50} value of **5000 nM**. Additionally, we assessed cell viability when HEK-293 cells were treated with sodium alginate nanoparticles and sodium alginate nanoparticles encapsulated with the Nus-OPH enzyme. In both cases, we observed approximately 80% cell viability with IC_{50} value of bare nanoparticles **10.57 ± 0.39 U/ml** and IC_{50} value of encapsulted nanoparticles **11.17 ± 0.09 U/ml**, indicating that these materials have minimal cytotoxic effects. This suggests that both types of nanoparticles may be safe for further applications in this context as shown in **Fig. 7.6.**

(A)



(B)

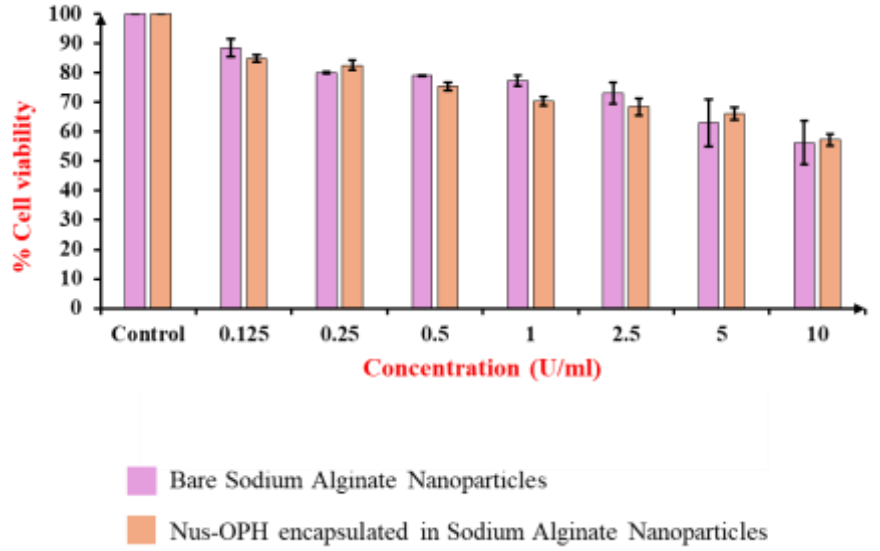


Fig. 7.6.: MTT cell viability assay of HEK-293 cells **(A)** when treated with EP. **(B)** when treated with Bare Sodium Alginate nanoparticles as well as Nus-OPH encapsulated in Sodium Alginate Nanoparticles.

7.2.4. Cellular uptake of sodium alginate nanoparticles

The results demonstrated that the FITC-tagged nanoparticles were effectively absorbed via endocytosis by the HEK-293 cells, confirming their ability to enter the cellular environment. This successful uptake suggests that these nanoparticles serve as the potential tool for detoxifying the harmful effects of organophosphate compounds inside the cell and emission profile of FITC tagged nanoparticles **(Fig. 7.7.).** By introducing these tagged nanoparticles, it may be possible to neutralize or remove these toxic compounds, thereby aiding in cellular protection and recovery. This finding could pave the way for further research into using similar nanoparticles for targeted drug delivery or detoxification applications.

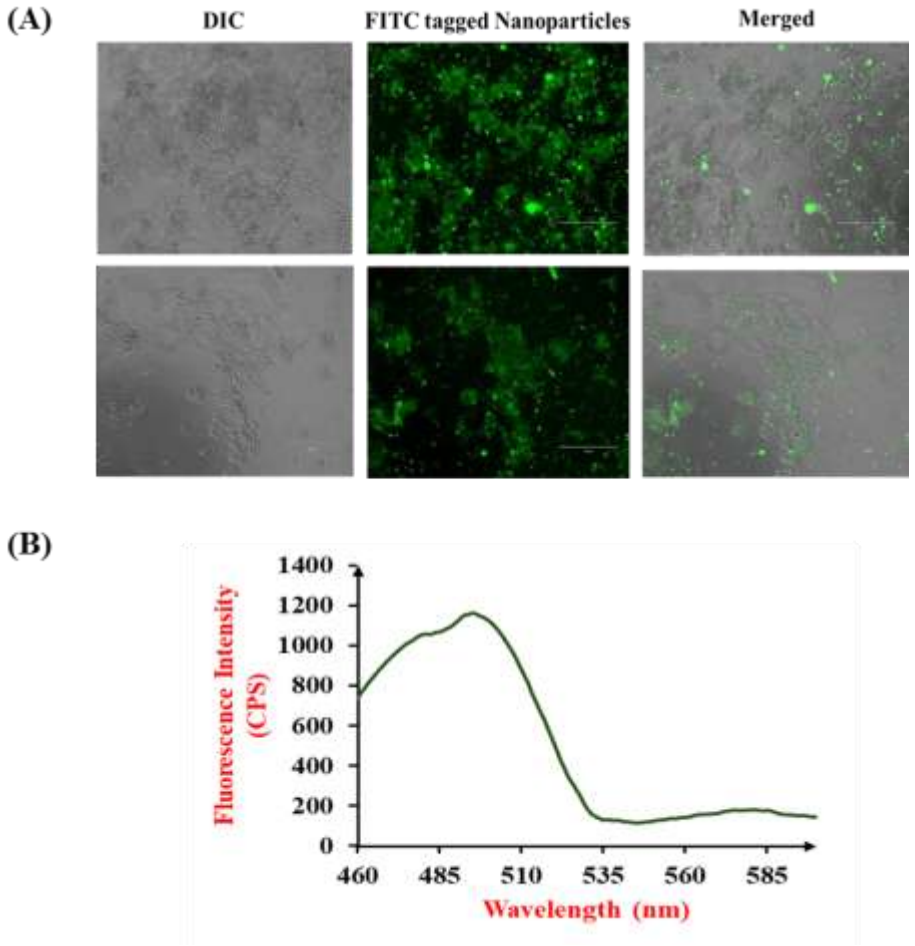


Fig. 7.7.: (A) Fluorescence microscopy images, shows the cellular uptake of the FITC tagged nanoparticles in HEK-293 cell line. **(B)** Emission profile at 519 nm of FITC tagged nanoparticles.

7.3. Summary

This study used sodium alginate nanoparticles encapsulating the Nus-OPH enzyme to break down organophosphate (OP) compound, specifically Ethyl Paraoxon (EP). Toxicity testing in HEK 293 cell lines demonstrated that enzyme-loaded nanoparticles successfully degraded OP chemicals, exceeding free enzymes. Cellular uptake of nanoparticles was detected, confirming intracellular OP breakdown. This method demonstrates the potential of nanoparticle-based enzyme delivery to detoxify OP chemicals in humans, particularly in cases of accidental inhalation. Organophosphate poisoning is a major global concern, causing symptoms ranging from respiratory distress to neurological abnormalities. This approach provides a promising and environmentally friendly repair strategy.

8. Conclusion

The successful expression and purification of enzymes such as Organophosphorous acid anhydrolase with FL mutation (OPAA-FL) and Organophosphorous hydrolase having Nus solubilization tag (Nus-OPH) causing degradation of organophosphate compounds. Bradford assay was done to determine the concentration of OPAA-FL and Nus-OPH enzyme. Activity assay of recombinant protein OPAA-FL and Nus-OPH was done. We saw successful degradation of Organophosphate compounds from enzyme loaded cotton fabrics. Type-A gelatin nanofibres is synthesized and carried out enzyme assay, observed enzyme reusability after multiple washes as well as degradation.

Sodium alginate nanoparticles encapsulating the OPH enzyme was done to showcase an advanced method for protecting and delivering the enzyme. The encapsulated OPH enzyme has proven to efficiently degrade Ethyl Paraoxon, highlighting its potential for practical applications in detoxifying or degrading organophosphate (OP) compound poisoning. Collectively, these methods highlight the potential of combining nanotechnology with enzymatic degradation to tackle the serious issue of organophosphate contamination. Various ways of degradation and detoxification of OP compounds have been explored. The experiment revealed that the cell viability of HEK-293 cells, when treated with Ethyl Paraoxon (EP), provided valuable insights into its toxicity. However, the use of a broad concentration range hindered the accurate determination of the IC-50 value, which will require a narrower concentration range in future experiments for precise calculation.

Additionally, the study showed approximately 80% cell viability when cells were treated with sodium alginate nanoparticles and sodium alginate nanoparticles encapsulated with the Nus- OPH enzyme, indicating low cytotoxicity. The successful cellular uptake of alginate Nanoparticles shows that it can be used to detoxify the effects of OP Compounds in humans. This highlights the potential of these sodium alginate-based nanoparticles could be used as safe materials in case of OP poisoning. This novel approach emphasizes a more refined approach to accurately measure toxicity and improve data reliability in future studies.

9. Future Work

The novelty in this research work lies in the use of Sodium Alginate nanoparticles to detoxify the cellular toxicity caused by organophosphate poisoning. The results have been shown that sodium alginate particles show cellular uptake. The study can further extent to see how these particles can detoxify the cells with high levels of OP compounds. The research could focus on use of different type of polymers to see and compare the activity of enzyme when cross-linked onto it while degrading OP compounds. Apart from using RGB analysis, some more ways could be explored to sense the OP compounds on the cloth itself.

APPENDIX-A

Pesticide	Permissible Limit	Guideline Issued By
Chlorpyrifos	0.03 µg/L	EU Drinking Water Directive
	0.1 µg/L	US EPA Drinking Water Maximum Contaminant Level
	0.5 µg/L	WHO Guideline Value
	0.7 µg/L	Canada Drinking Water Quality Guidelines
Diazinon	0.02 µg/L	EU Drinking Water Directive
	0.3 µg/L	US EPA Drinking Water Maximum Contaminant Level
	0.03 ppm (soil)	US EPA
Malathion	0.02 µg/L	EU Drinking Water Directive
	0.2 µg/L	US EPA Drinking Water Maximum Contaminant Level
	2 ppm (soil)	US EPA
Parathion	0.0002 µg/L	EU Drinking Water Directive
	0.006 µg/L	US EPA Drinking Water Maximum Contaminant Level
	0.09 ppm (soil)	US EPA

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